UNDERSTANDING THE INTERACTION BETWEEN *MYCOPLASMA BOVIS* AND BOVINE RESPIRATORY MACROPHAGES

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By

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ABSTRACT

Mycoplasma bovis is the most pathogenic bovine mycoplasma in Europe and North America. It forms an important component in the bovine respiratory disease (BRD) complex, a multifactorial disease of feedlot cattle that causes major economic loss. Naturally occurring *M. bovis* persist in a herd over an extended period and can be consistently identified not only in lesions but also commonly in healthy lungs and those with pneumonia indicating that *M. bovis* is capable of persisting in lungs of recovered animals. This suggests that *M. bovis* can modulate alveolar macrophage effector functions, although the mechanisms employed by *M. bovis* are not well understood. In this thesis, I determined how *M. bovis* modulates bovine alveolar macrophage (BAM) effector functions. I used primary alveolar macrophages infected with *M. bovis* Mb1 to demonstrate that the production of nitric oxide and cytokines was impaired. I observed a reduction in the production of nitric oxide but not a decrease in gene transcription of iNOS; and no stimulation of the pro-inflammatory cytokine TNF-α coupled with an increase of IL-10 expression in infected BAMs. These results suggest modulation of the immune response by *M. bovis*. To better understand other strategies of immune suppression by *M. bovis* to evade the host immune responses, I focused on the ability of *M. bovis* to modulate macrophage apoptosis during infection. Previously, studies on *M. bovis* infection have reported it to exert both pro- and anti-apoptotic effects on a diversity of cell types, including neutrophils, lymphocytes, monocytes and macrophages. In this thesis, I sought to understand the mechanism of inhibition of apoptosis using a BoMac bovine macrophage cell line to identify the apoptosis pathways modulated by *M. bovis* infection. The results indicate that *M. bovis* strain Mb1 is able to delay STS-induced apoptosis in BoMac cells, activate the NF-kβ pathway and induce up-regulation of the pro-survival genes Bcl-2 and Bcl-X_L. Additionally, upon infection *M. bovis* inhibits the activity of caspases 3, 6, and 9, reduces ROS production and inhibits DNA fragmentation. These results support the observation that *M. bovis* inhibit apoptosis for survival and potentially facilitate bacterial survival, replication and transmission.

The results presented in this thesis taken together detail how *M. bovis* modulates the macrophages effector functions and apoptosis to survive within the bovine host. The studies also improve the understanding of factors that contribute to virulence; dissemination and immune evasion of a pathogen is critical knowledge to contemplating new vaccines and therapeutics.

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LIST OF ABBREVIATIONS

AIF: Apoptosis inducing factor

Apaf-1: Apoptotic protease activating factor

Arg1: Arginase 1

ATP: Adenosine triphosphate

BAD: BCL2 antagonist of cell death

BAL: Broncho-alveolar lavage fluid

BAMs: Bovine alveolar macrophages

BAX: BCL2 associated X protein

BCL-2: B-cell lymphoma 2

Bcl-XL: BCL2 related protein, long isoform

BHV-4: Bovine herpesvirus-4

BIM: BCL2 interacting protein BIM

BIR: Baculovirus IAP repeat motifs

BSA: Bovine serum albumin

c-FLIP: FLICE-inhibitory protein

C: Cytosine

CAD: Caspase-Activated DNAse

CARDS: Community-Acquired Respiratory Distress Syndrome

Caspase: Cysteinyl aspartic acid-protease

CBPP: Contagious bovine pleuropneumonia

CD: Cluster of differentiation

CFSE: Carboxyfluorescein N-hydroxysuccinimidyl ester

ConA: Concanavalin A

D-MEM: Dulbecco's modified eagle medium

DAMP: Damage-associated molecular pattern

DGGE: Denaturing gradient gel electrophoresis

DISC: Death-inducing signalling complex

DNA: Deoxyribonucleic acid

EBL cell: Embryonic bovine lung

EBTr cells: Embryonic bovine tracheal cells

EDTA: Ethylene diamine tetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

FACS: Flow cytometry

FADD: Fas-associated death domain

Fas: Fatty acid synthetase

FasL: Fatty acid synthetase ligand

FSC-A: Forward scatter area

G: Guanine

GIT: Gastro-intestinal tract

H&E: Haematoxylin and eosin

H_2O_2: Hydrogen peroxide

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IAP binding protein with low PI

IAP: Inhibitor of apoptosis proteins

IBMs: IAP-binding motifs

ICE: Integrative conjugative element

IFN-γ: Interferon gamma

Ig: Immunoglobulin

IL: Interleukin

IM: Intramuscularly

iNOS: Inducible nitric oxide synthase

IS: Insertion sequence

kDa: Kilodaltons

LAM: Lipoarabinomannan

LPO: Lipo-oligosaccharides

LPS: Lipopolysaccharide

Mcl-1**:** Myeloid leukemia cell differentiation protein

MHC: Major histocompatibility complex

MLKL: Mixed lineage kinase domain

MLST: Multilocus sequence typing

MLVA: Multiple locus variable number tandem repeat analysis

NEAA: Non-essential amino acids

NF-kβ: Nuclear Factor kappa beta

NK: Natural killer cells

NO: Nitric oxide

OVA: Ovalbumin

PAMPs: Pathogen-associated molecular patterns

PARPs: Pattern recognition receptors

PBMC: Peripheral blood mononuclear cell

PBS-BN: PBS, 1% BSA, 0.05% Na-Azide, pH 7.4

PBS: Phosphate buffered saline

PBST-G: PBS-T + 0.5% gelatin

PBST: PBS + 0.10% Tween 20

PCD: Programmed cell death

PCR: Polymerase chain reaction

PD1: Programmed cell death protein 1

PFGE: pulsed-field gel electrophoresis

PI: Propidium iodide

PI3K/Akt: Phosphatidylinositol 3-kinase/Akt

qPCR: Quantitative PCR

RBCs: Red blood cells

RFLP: Restriction fragment length polymorphism

RIPK3: Receptor-interacting serine/threonine-protein kinase

RNA: Ribonucleic acid

ROS: Reactive oxygen species

rRNA: Ribosomal ribonucleic acid

RT: Room temperature

SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Smac/DIABL: Second mitochondrial activator of caspases/direct

SSC-A: Side scatter area

STS: Staurosporine

TCR: T-cell receptor

TE: Tris-EDTA

T_H: Helper T-cell

TLR: Toll like receptors

TNF-α: Tumor necrosis factor alpha

TNFR-1: Tumor necrosis factor receptor-1

TRADD: TNF receptor-associated death domain

USA: United States of America

UT: Urinary tract

UV: Ultra violet

V-ZAD-FMK: Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone

γδ-T-cells: Gamma delta T-cells

CHAPTER 1. Introduction and literature review

1.1. Mycoplasmas

Classification

Mycoplasma bacteria belong to the class Mollicutes in the phylum Firmicutes consisting of the genus Mycoplasma, Phytoplasma and Ureaplasma. Phylogenetic analysis of 16S RNA oligonucleotide sequences indicates that the Mollicutes evolved by reductive evolution from Gram-positive eubacteria, as reflected by their low genetic guanine-plus-cytosine content (G+C) content of 23-40 mol% (Woese et al., 1980).

Structure

Mycoplasmas are the simplest smallest independently replicating bacteria. They are minimalist cells with a few ribosomes, soluble proteins, a plasma membrane and a double-stranded circular deoxyribonucleic acid (Dudek et al.) molecule. Some mycoplasmas have capsules that are essential for survival (Razin et al., 1998). Mycoplasmas lack a cell wall and their plasma membrane is composed of membrane proteins, phospholipids, lipoproteins, glycolipids and lipoglycans. The lack of a cell wall confers unique characteristics: Staining with the Gram stain is not effective, and they are resistant to antibiotics that target the peptidoglycan synthesis such as beta-lactams. Moreover, they are also intrinsically resistant to Rifampin, an antimicrobial that targets ribonucleic acid (RNA) synthesis (Razin and Hayflick, 2010).

Mycoplasmas encode surface exposed lipoproteins that are good antigenic determinants and this property is explored for diagnostics of infections. Mycoplasmas are pleomorphic as they can change their shapes due to lack of an internal cytoskeleton. The majority are circular in nature with a diameter of about 0.2 to 0.8μm, while others are pear-shaped, flask-shaped or rod-like, occurring in coccus and filamentous forms (Razin and Hayflick, 2010). The small sizes of mycoplasmas allow them to pass through 0.45μm sterilization filters making it a nuisance laboratory contaminant of cell cultures.

Most mycoplasmas are non-motile and have no flagella. However, some of the flask-shaped mycoplasma species, including the human and animal pathogens (*M. pneumoniae*, *M. genitalium*, *M. gallisepticum*, *M. pulmonis,* and *M. mobile*) glide on liquid-covered surfaces without the aid

of extracellular structures (Jaffe et al., 2004; Pich et al., 2006). Due to lack of locomotive structures and a cell wall, it is reasonable to conclude that the cell membrane is involved in attachment and gliding. The gliding mechanism is always toward the terminal organelle head-like protrusions with the involvement of adhesin proteins. In *M. pneumoniae*, the protein P1 (Feldner et al., 1982), GapA of *M. gallisepticum* (Goh et al., 1998), MgPa of *M. genitalium* (Inamine et al., 1989), and Gli349 of *M. mobile* (Uenoyama et al., 2004) are involved in gliding.

Habitat

As reviewed by Pitcher *et al*., mycoplasmas cause disease in plants, human, and animals. In animals, they have been isolated from mammals, birds, reptiles, amphibians, and fish revealing unique species (Pitcher and Nicholas, 2005).

Multiplication and cell invasion

Mycoplasmas possess a circular chromosome ranging in size between 0.58 to 2.20Mb. Due to their small genomes, they lack essential genes for biosynthesis of numerous compounds and rely on the host biosynthetic activity and metabolism for survival (Rottem, 2003). The lack of certain metabolic genes and reliance on host nutrients also makes them fastidious organisms that are difficult to cultivate in a laboratory setting and grow very slowly. Mycoplasma culture medium must contain serum to provide sterols and cholesterol for plasma membrane synthesis (Dahl, 1993). Mycoplasmas also use the universal stop codon, UGA as a tryptophan codon (Razin et al., 1998). In general, the presence of the UGA codon in mycoplasma genes hinders expression of proteins in commonly used *Escherichia coli* expression systems leading to premature truncation of the target proteins.

Some mycoplasmas invade cells using adhesins. These are mostly surface proteins that facilitate adhesion and have an effect on invasion (Razin and Jacobs, 1992; Rottem, 2003; Sachse et al., 1996). They enter the cell through a site-receptor mediated event or phagocytized. The complex interaction between mycoplasmas and the host immune system induces both pathogen-specific and non-specific immune responses. Their ability to display antigenic variation and host immune system antibody modulation contribute to disease pathogenesis and avoidance of clearance by the host (Razin and Jacobs, 1992).

Other virulence factors involved include competition for and depletion of nutrients in host cells. Some mycoplasma species hydrolyse arginine to citrulline as a major energy source depriving the host cell of an essential amino acid for host maintenance and a substrate for NOS for antimicrobial function. Infection of host cells by mycoplasmas also leads to stimulation of pro-inflammatory cytokines, cytotoxicity and activation of immune cells through secretion of toxins, and surface antigens contribute to the disease pathogenesis (Rottem, 2003).

As reviewed by Christodoulides *et al*., mycoplasma surface lipoproteins play a role in in the immune modulation of the host (Christodoulides et al., 2018). In *M. fermentans,* the MALP (Macrophage-activating lipopeptide) lipoprotein elicited a strong inflammatory response in macrophages through the activation of toll like receptors, TLR-2 and 6 (Muhlradt and Frisch, 1994; Muhlradt et al., 1997). The production of Community-Acquired Respiratory Distress Syndrome (CARDS) toxin an ADP-ribosylating and vacuolating protein by *M. pneumoniae and M. penetrans* cause severe cytopathology in mammalian cells (Johnson et al., 2009; Kannan and Baseman, 2006). Other virulence factors described include hemolysins (Kannan and Baseman, 2000; Somerson et al., 1965) and endonucleases (Bendjennat et al., 1997).

Human mycoplasmas

Human mycoplasmas cause a variety of diseases and are mostly opportunistic pathogens. The most common pathogen is *M. pneumoniae* that causes atypical pneumonia and tracheobronchitis a disease of the upper respiratory tract (Cassell et al., 1987). In contrast, *Ureaplasma spp.*, *M. genitalium*, *M. hominis,* and *M. penetrans* are sexually transmitted mycoplasmas that cause pelvic inflammatory disease, pyelonephritis, and nongonococcal urethritis often, though not always, in immune compromised patients infected with human immunodeficiency virus (Blanchard and Montagnier, 1994; Cassell et al., 1987). Other mycoplasmas that are found in the mouth and throat as normal flora include *M. orale, M. salivarium,* and *M. buccale.*

Animal mycoplasmas

The range of animal hosts that are infected by mycoplasmas vary from domesticated animals, wildlife, birds, and fish (Nicolet, 1996). It has long been appreciated that most *Mycoplasma spp*. exhibit extreme host specificity except for a few documented cases of crossover between related host such as ruminants (Pitcher and Nicholas, 2005). In animals, mycoplasmas are mostly found

as commensals on the mucous membrane of the respiratory tract, gastro intestinal tract (GIT), urinary tract (UT), joint surfaces, and in bovine mammary glands. They are mostly opportunistic pathogens that cause infections and diseases in immune-compromised host (Razin et al., 1998). The majority of the pathogenic mycoplasmas in livestock cause chronic diseases with high morbidity causing massive losses in the food production industry as summarised in the table 1.1 below.

Table 1. 1: Selected mycoplasma species of veterinary importance.

Host	Organism	Disease	Reference
Cattle	*M. mycoides subsp. mycoides (Mmm)*	CBPP (contagious bovine pleuropneumonia)	(Cottew and Yeats, 1978)
	M. bovis	Polyarthritis, mastitis, abortion, sterility, pneumonia, kerotoconjunctivitis,	(Pfutzner, 1990)
	M. dispar	Pneumonia	(Gourlay and Leach, 1970)
	M. leachii	Arthritis, mastitis	(Manso-Silvan et al., 2009)
Bison	*M. bovis*	Pneumonia, abortions, necrotic pharyngitis	(Dyer et al., 2008)
Sheep	*M. agalactiae*	Contagious agalactia	(Manso-Silvan et al., 2012)
	M. ovipneumoniae	Pneumonia	(Alley et al., 1975)
Goat	*M. capricolum subsp. capripneumoniae*	Contagious caprine pleuropneumonia	(Thiaucourt and Bolske, 1996)
	M. mycoides subsp. capri (Mmc)	Contagious agalactiae/pneumonia	(Manso-Silvan et al., 2009)

	M. capricolum subsp. capricolum	Contagious agalactiae/pneumonia	(Thiaucourt and Bolske, 1996)
Swine	*M. hyopneumoniae*	Porcine enzootic pneumonia	(Vasconcelos et al., 2005)
	M. suis	Hemolytic anemia	(Ross, 1973)
	M. hyorhinis	Pneumonia, arthritis	(Ross, 1973)
Poultry	*M. synoviae*	Pneumonia Synovitis	(Vasconcelos et al., 2005)
	M. gallisepticum	Chronic Respiratory disease	(Ley et al., 1993)

Table compiled by the author.

1.2 *Mycoplasma bovis*

The genome size of *M. bovis* is approximately 948 kbp (Li et al., 2011) to 1,038 kbp (GenBank: LT578453.1), and has a G+C ratio of 27.29% (Chen et al., 2017; Razin et al., 1998). *Mycoplasma bovis* is an important pathogen that causes various diseases in animals that include pneumonia (Caswell and Archambault, 2007; Gagea et al., 2006a), mastitis (Jasper et al., 1987), arthritis (Gagea et al., 2006a; Kumar et al., 2011), infertility (Hermeyer et al., 2012b), and keratoconjunctivitis (Alberti et al., 2006). It is also becoming clear that *M. bovis* is a key player in the BRD complex worldwide (Caswell et al., 2010; Maunsell and Donovan, 2009; Nicholas et al., 2008). *M. bovis* is considered the most pathogenic bovine mycoplasma in Europe and North America (Nicholas, 2011), and if not treated early enough, *M. bovis* infection might be fatal especially in calves that have a weak immune system. Even further, the pathogen is known to affect the productivity of beef cattle with the challenges being prevalent in various parts of the world (Kumar et al., 2011; Nicholas, 2011). Notably, the infection is also hard to identify due to lack of consistent manifestation of the disease, which can require specific diagnosis approaches to determine, which might impact farmers, particularly those with large herds.

Epidemiology

M. bovis can be introduced into beef herds after co-mingling of asymptomatic infected animals into the herd (Nicholas and Ayling, 2003). Bovine mycoplasmosis is prevalent in most parts of the world. In the feedlot industry *M. bovis* is estimated to cost across Europe €144 million per year (Nicholas and Ayling, 2003), and in the US $32 million per year in feedlot cattle and $108 million per year in dairy cattle due to mastitis (Maunsell et al., 2011). *M. bovis* occurs both as normal flora and opportunistic pathogen in cattle (Maunsell et al., 2011). In Ontario, Allen *et al*. examined broncho-alveolar lavage fluid (BAL) samples and reported 48% recovery of *M. bovis* in the control group (calves that started the study with a clinical score of zero) with no BRD and 60% of recovery in BRD cases on day one at the research facility and after 12 days, they observed that a high proportion of feedlot calves were exposed to *M. bovis* with 80% recovery from the control group (calves that started with a clinical score zero) and 100% from the BRD cases (Allen et al., 1991). In another study in 3 Canadian feedlots, 28% of the necropsied cattle had CPPS (chronic pneumonia and polyarthritis syndrome), a disease mainly attributed to *M. bovis* (Maunsell et al., 2011). In North American bison, *M. bovis* was reported to cause up to 45% morbidity and case mortality (Sweeney et al., 2013). In addition, cases of pneumonia (Dyer et al., 2008), abortions (Register et al., 2013) and necrotic pharyngitis (Dyer et al., 2013) have been recently described caused by *M. bovis* in bison. The pathogen is known to colonize its hosts and can survive without the host expressing any symptoms making observation of sickness a challenge (Nicholas and Ayling, 2003; Pfutzner, 1990). Infected animals shed the pathogen via the nasal secretions, nose-to-nose contact (Maunsell et al., 2011); contaminated milk (Aebi et al., 2012), and infected semen (Pfutzner, 1990). Moreover, *M. bovis* is spread from farm to farm through borrowing of bulls, poor boundary fence, shared grazing or housing, which indicates the prevalence rates are high and tends to increase during transportation of animals from one region to the other, thus leading to high infection rates (Castillo-Alcala et al., 2012; Nicholas and Ayling, 2003; Timsit et al., 2012).

Pathogenesis and virulence

Some mycoplasma biological characteristics are of great importance in their clinical role in the disease. A small genome means that mycoplasmas relay on the host for its metabolic requirement sand the lack of a cell wall makes them resistant to beta-lactam antimicrobials (Caswell et al.,

2010). The respiratory tract and the mammary glands are the most affected parts that result in shedding and persistence of *M. bovis*. Clinical studies of BRD show that naturally and experimentally infected calves had pulmonary lesions with characteristic coagulative necrosis (Thomas et al., 1986) surrounded by inflammatory cells such as macrophages and neutrophils (Rodriguez et al., 1996). The lesions found in the lungs are bilateral and involve 20-90% of the lung with characteristic multiple round foci of caseous necrosis. The affected lungs are deep red and consolidated (Caswell et al., 2010). Once transmitted, entry into the body leads to colonization that lead to disease progression if left untreated. We still have a limited understanding of the *M. bovis* virulence factors and the mechanisms of pathogenicity as disease development often is due to synergism to co-infection with other pathogens. For a long time, limited research techniques and lack of genetic tools hindered host-pathogen interaction studies. Tools such as random transposon mutagenesis have been used recently to create *M. bovis* mutants (Sharma et al., 2014) and this will allow future studies of host-pathogen interactions. A number of pathogenic mechanisms of *M. bovis* have been identified that have an effect on the infected host.

Variable surface proteins

Due to lack of a cell wall, lipoproteins are readily exposed on the mycoplasma membrane and act as key antigens (Behrens et al., 1994; Lysnyansky et al., 1996). As argued by Behrens *et al*., mycoplasmas posses cell surface lipoproteins that play a key role in inducing pro-inflammatory cytokines and, hence contributing to the disease pathogenesis (Behrens et al., 1994). Like other mycoplasma species (Razin et al., 1998) size variation and phase variation (ON-OFF) of surface lipoproteins occur in *M. bovis*. Variable surface proteins (Vsps) found on the cell surface are subject to modification, presenting different antigenic mosaic repertoires due to high-frequency DNA-rearrangement in a population (Lysnyansky et al., 1996; Lysnyansky et al., 1999; Sachse et al., 2000). This switching was also demonstrated *in vivo* in experimentally infected calves (Buchenau et al., 2010). Thirteen single copies of *vsp* genes have been identified in *M. bovis* strain PG45 with a conserved N-terminal domain for membrane attachment and a variable C-region that is exposed. Mycoplasmas use DNA recombination to produce a mosaic of Vsps (Lysnyansky et al., 1999). The Vsps are used for colonization, adhesion, and immune evasion from the host immune system (Behrens et al., 1994). *M. bovis* successfully uses high-frequency

antigenic switching strategy to maintain strain population diversity in an attempt to evade the host immune system hence, contributing to the chronic presentation of the disease (Buchenau et al., 2010). Other studies are required to determine more roles of the variable surface antigens in the disease.

Adhesion

Once transmitted, entry into the host cells requires adherence, a critical step to facilitate colonization and infection of the lung (Sachse et al., 1996). In *M. pneumoniae*, a tip like structure containing adhesin P1 is used for attachment (Razin and Jacobs, 1992). On the contrary, there is no tip-like structure in *M. bovis* (Behrens et al., 1996) but the surface membrane proteins are thought to be involved in adhesion (Behrens et al., 1996; Razin and Jacobs, 1992). In a Western blot adhesion assay using embryonic bovine lung cells (LeBlanc et al.), the surface membrane protein p26 was demonstrated to be an adhesin of *M. bovis* (Sachse et al., 1996) and some members of the Vsps, VspA, VspB, VspE, and VspF (Sachse et al., 2000). In another study, primary bovine bronchial epithelial cells were used to characterise the cytoadherence properties of *M. bovis* strain PG45 (Thomas et al., 2003b). Significant variation in the cytoadherence among different host cells tested and different pathogenic strains were also observed (Thomas et al., 2003a; Thomas et al., 2003b), with the less pathogenic strains having lower adherence rates.

Cell invasion

Using immunohistochemistry (IHC) and electron microscopy *M. bovis* antigens were detected in different host cells such as macrophages, neutrophils, bronchiolar epithelial cells, monocytes, and lymph nodes of infected calves (Adegboye et al., 1995; Kleinschmidt et al., 2013; Maeda et al., 2003; Rodriguez et al., 1996). In primary embryonic calf turbinate cells, an intracellular stage of *M. bovis* was demonstrated using electron microscopy (Burki et al., 2015). Moreover, Van der Merwe *et al.,* also reported intracellular *M. bovis* in peripheral blood mononuclear cell (PBMC) and red blood cells (RBCs) and this was later followed by reports of invasion in embryonic bovine tracheal cells (EBTr) and embryonic bovine lung (Burki et al., 2015) cell lines, primary alveolar macrophages, and the BoMac cell line using the gentamicin protection assay (Burgi et al., 2018; Burki et al., 2015; Maina et al., 2019; Suleman et al., 2016a; van der Merwe et al., 2010).

The ability of *M. bovis* to invade and persist in both phagocytic and non-phagocytic cells may play a major role in the dissemination of the pathogen to different sites, escape from elimination by the host immune system and antimicrobial therapy and overall contributing to the pathogenesis of the bacteria and progression of bovine mycoplasmosis.

Biofilm formation and production of secondary metabolites

Biofilm are known to harbour pathogens and aid in persisting infections leading to the chronic manifestation of the disease (Mah and O'Toole, 2001). In mycoplasmas, biofilm formation occurs independently of their pathogenicity and their capacity to form biofilms is reliant on their ability to adhere the host cells. Due to the presence of highly variable Vsps and their involvement in adherence, there is also a variable ability to form biofilms depending on the Vsp patterns (McAuliffe et al., 2006; Sachse et al., 1996). Even though most mycoplasmas do not posses biofilm formation genes found in other bacterial species McAuliffe *et al*., showed that *M. bovis* was resistant to heat and desiccation, Further studies are necessary to investigate the formation of biofilms *in vivo* (McAuliffe et al., 2006).

Previous reports show that production of hydrogen peroxide (H_2O_2) is an important virulence factor in different mycoplasma *spp*. such as *Mycoplasma mycoides subsp. mycoides* (Pilo et al., 2005). Similarly, mycoplasma H_2O_2 production was observed in all the *M. bovis* isolates used in another study (Schott et al., 2014) and *in vitro* passage studies showed a decrease in H_2O_2 production with an increase in passage attributed to the loss of a 32-kDa protein during passage (Khan et al., 2005). Unlike *M. pneumoniae* where glycerol metabolism plays a key factor in cytotoxicity, there was no indication of glycerol metabolism in any of the *M. bovis* strains tested as there was no $_L$-α-glycerophosphate oxidation (Hames et al., 2009; Khan et al., 2005).

Modulation of immune responses to *M. bovis*

Introduction

Previous reports infer that elements of both innate immunity as well as adaptive responses play a significant role to *M. bovis* infections and pathology (Bennett and Jasper, 1977; Howard et al., 1986). It has been reported that after *M. bovis* immune responses, infection are skewed toward a TH_2 phenotype (moderated interferon gamma (IFN-γ) and high interleukin, IL-4 levels in T-cells,

high IgG1, low IgG2a titres in the serum, and high IL-10 levels in monocytes) (Mulongo et al., 2014; Vanden Bush and Rosenbusch, 2003). Moreover the alteration of surface antigens is also an important aspect of the immune modulation by *M. bovis* infection (Rosengarten et al., 1994). However, despite all of this information the immune response that confers protection are still largely unknown.

Innate immunity responses to *M. bovis*

During invasion of the respiratory tract, mycoplasmas encounter non-specific immune responses from the host immune cells. Jungi *et al*., suggests that alveolar macrophages effector functions are modulated upon infection with mycoplasmas and their inappropriate activation can promote an excessive inflammatory response (Jungi et al., 1996). They also demonstrated that induction of NO, TNF-α, and procoagulant activity was strain-specific giving variable differential induction profiles (Jungi et al., 1996). Activation of macrophages leads to the recruitment of neutrophils to the site of infection (Gagea et al., 2006b). *In vitro* studies of bovine neutrophils, infection with *M. bovis* strain Mb1 reported an increase in elastase release, inhibition of NO production, along with stimulation of pro-inflammatory cytokines TNF-α and IL-12 (Jimbo et al., 2017). Similarly, *M. bovis* inhibited neutrophil respiratory burst (Thomas et al., 1991). In monocytes, infection with *M. bovis* strain Mb1 caused an immune suppression of IFN-γ and TNF-α, pro-inflammatory cytokines and an up-regulation of anti-inflammatory cytokine IL-10 (Mulongo et al., 2014).

The TLRs are expressed on phagocytic cells such as dendritic cells, neutrophils and macrophages in the lungs and are a vital component of the innate immune responses at these sites. They recognise pathogen-associated molecular patterns (PAMPs) derived from pathogens. To date, 10 TLR bovine genes are characterized (Menzies and Ingham, 2006). Evidence in human mycoplasmas shows that surface lipoproteins are involved in the induction of TLR-2 and 6 during mycoplasma invasion of monocytes (Into et al., 2004a), and mRNA levels of TLR-2 and 4 in bovine mammary epithelial cells stimulated with heat-killed *M. bovis* were significantly increased (Gondaira et al., 2018).

Humoral immune responses against *M. bovis*

Since *M. bovis* is considered an extracellular respiratory pathogen, local antibodies responses play a significant role in attempting to inhibit the attachment and growth of the pathogen, in the

host cells. A previous study characterized serum antibody responses against surface-exposed Vsps lipoproteins of *M. bovis* (Rosengarten et al., 1994). Different experimental infection studies of calves reported a strong production of antigen-specific IgG responses to *M. bovis* (Howard et al., 1986; Vanden Bush and Rosenbusch, 2003) and predominantly IgG1 (Hermeyer et al., 2012a; Vanden Bush and Rosenbusch, 2003). The low IgG2 response is thought to contribute to pathogenesis, as there is less opsonisation and poor immunity (Vanden Bush and Rosenbusch, 2003). Other investigators have described opsonisation of *M. bovis* in bovine alveolar macrophage as crucial in the killing of the pathogen (Howard et al., 1976).

Cell-mediated immune responses to *M. bovis*

M. bovis infection induces cell-mediated immune responses (Bennett and Jasper, 1977) and using an IL2R-α subunit (CD25) as an indicator of T-cell activation, Vanden Bush reported the different subsets of T-cells (CD4 and γδ-T cells) that were activated during the recall along with production of moderate IFN-γ and high IL-4 (Vanden Bush and Rosenbusch, 2003). Down-regulation of lymphocyte proliferation is another important immunosuppression strategy of *M. bovis* infection (Mulongo et al., 2013; Prysliak et al., 2013; van der Merwe et al., 2010; Vanden Bush and Rosenbusch, 2003). We suggested that the expression of programmed cell death protein 1 (PD-1) and its ligand PD-L1 played a role in the impairment of antigen-specific T-cell responses (Suleman et al., 2018). The PD-1 receptor is expressed on activated T-cells and recognizes its ligand, PD-L1 expressed on activated dendritic and macrophages cells and engagement of both leads to poor immune responses (Keir et al., 2008). In the study, we used EBTr cells, EBL cells, and BAMs cells to report an increase of PD-L1 after *M. bovis* infection and demonstrated restoration of *M. bovis* infected PBMC proliferation using blocking anti-PD-1 antibodies. These data can inform vaccine strategies to overcome the PD-1-related immune exhaustion of T-cells during *M. bovis* infection (Suleman et al., 2018).

Diagnostics

Since most *M. bovis* infections are multifactorial, clinical and pathological signs cannot be used for diagnostics, and the sample choice depends on the disease suspected. In BRD and pneumonia, nasal swabs, BAL, serum and affected lungs can be collected. A study by Thomas *et al*., showed that BAL was a better choice of clinical samples in suspected lower respiratory tract infections

(Thomas et al., 2002) than nasal swabs. The diagnosis of the disease is performed using various methods that include testing for antibodies using the indirect enzyme-linked immunosorbent assay (ELISA). Serology is particularly important in cases where extensive antibiotic use in the herd is practiced as this severely hinders isolation of viable mycoplasma by culture (Parker et al., 2018). However, the disease is difficult to detect in natural infections since studies have shown that the individual titres do not correlate with infection (Le Grand et al., 2002). Nonetheless, the use of ELISA is useful in group testing since the rate of seroconversion is high which is indicative of the presence of the pathogen among the affected herd (Kumar et al., 2011).

Traditionally, in clinical samples, the diagnostic process is usually complicated since it required isolation of culture with *M. bovis* growth generally occurring within 2-6 days at 37°C and 5% CO_2 before the sample is determined as negative. It is a fastidious organism and requires supplementation of its medium with cholesterol, serum, and DNA and appearance of 'fried egg' morphology of the colonies under a microscope is confirmatory of mycoplasma growth on solid medium (Razin and Jacobs, 1992).

Immunohistochemistry can also be used to detect *M. bovis* antigens in frozen, fresh or formalin-fixed paraffin-embedded tissues. Localised antigens close to the necrotic tissues suggest causation of the infection (Adegboye et al., 1995; Gagea et al., 2006a; Yilmaz et al., 2016).

Molecular diagnosis of *M. bovis* is more efficient, specific, and sensitive from various clinical samples compared to culture methods (Sachse et al., 1993). Currently, polymerase chain reaction (PCR) assays that are designed to detect *M. bovis* target the 16S Ribosomal ribonucleic acid (rRNA) genes followed by separation of the PCR products using denaturing gradient gel electrophoresis (DGGE), this approach is useful in detecting mixed cultures (Jozefova et al., 2014). The disadvantage of targeting the 16S rRNA genes of *M. bovis* is the cross-amplification seen with closely related mycoplasmas such as *M. agalactiae* (Gonzalez et al., 1995). Other target genes have been used as quantitative PCR (qPCR) fluorescent reporter probe for greater specificity that allows multiplexing in a single reaction such as, the use of the *uvrC* gene (Clothier et al., 2010; Rossetti et al., 2010) and *oppD/F* genes (Sachse et al., 2010) for *M. bovis* diagnostics.

The challenges of using PCR and culture methods in diagnostics is that they both rely on shedding of the organism at the time of sampling, hence the sensitivity of the clinical samples is quite low due to uneven distribution of *M. bovis* in the diseased samples, the intermittent and shedding nature of the disease in the affected animal and mishandling of the sample (Parker et al., 2018). ELISA can prove to be complementary as it can measure past exposure to pathogen.

The tools for molecular typing of mycoplasmas include pulsed-field gel electrophoresis (PFGE), multiple locus variable number tandem repeat analysis (MLVA), and multilocus sequence typing (MLST). In PFGE, the chromosomal DNA is digested using restriction enzymes and the resulting DNA fragments are separated in an agarose gel producing band patterns specific to different strains of *M. bovis* (Arcangioli et al., 2012). In MLST, analysis for sequence uniqueness using multiple housekeeping genes within the genome is performed and based on the differences, the relatedness of the isolate is determined (Rosales et al., 2015), however, in MLVA the analysis is based on differences in the number of tandem repeats at a selected gene (Register et al., 2014,Spergser, 2013 #5413).

To date the completed whole genome sequences of eleven *M. bovis* isolates are available with five *M. bovis* isolates being used in comparative genomics analysis (Chen et al., 2017; Li et al., 2011; Qi et al., 2012; Sun et al., 2018; Wise et al., 2011). Although mostly conserved, the genomes have varied restriction-modification genes, variable numbers and copies of IS and invasion of different integrative conjugative element (ICE) that are important for traceability. Using comparative genomics, these sequences can be further used to identify conserved candidate antigens for vaccine candidates, disease surveillance and diagnostics.

Treatment

The treatment and control of *M. bovis* are challenging since they lack a cell wall making beta-lactam antimicrobials not effective. Similarly, they are intrinsically resistant to rifampicin and sulphonamides, as mycoplasma does not synthesise folic acid. In contrast, mycoplasmas are generally susceptible to antimicrobials that affect the integrity of the cell membrane: ionophores; protein synthesis: tetracycline, macrolides, and DNA synthesis: fluoroquinolones. There have been studies of unresponsive antimicrobial treatment in cattle with BRD (Haines et al., 2001a) along with 80% isolation of *M. bovis* in the lungs and joints of the unresponsive feedlot cattle. In

Europe drugs such as oxytetracyclines, tilmicosin, and spectinomycin have also started showing resistance (Ayling et al., 2000).

There are other antibiotics that can be used to manage the disease. In the United States tulathromycin and florfenicol are the only two antimicrobials approved (Godinho et al., 2005). Experimental trials conducted on affected calves have shown that the animals showed low temperatures and lower lung lesions, demonstrating the effectiveness of these antibiotics. Nonetheless, the efficacy of the drugs is not 100% requiring the adoption of other methods to ensure that affected animals recover fully (Maunsell et al., 2011). A Western Canadian bison study reported higher MIC values for tetracyclines and spectinomycin in clinical bison isolates compared to Canadian beef feedlots or dairy cattle herds isolates. Also, all the bison isolates tested in the study were resistant to tilmicosin and resistant to tulathromycin (Suleman et al., 2016b). This study described marked differences between clinical bison isolate susceptibility profiles to other cattle isolate studies performed (Suleman et al., 2016b).

Control

The management of *M. bovis* infection is undertaken using various approaches that include close herding where the herds are managed more strictly without buying and borrowing bulls from other farms, proper fencing and lack of community grazing, which limits the spread of the disease. Further, the screening of newly purchased animals is critical to the prevention spread since affected animals can be quarantined, thus ensuring the healthier ones are not affected (Maunsell et al., 2011). In particular, the farmer should assess the health records of calves to establish if incidences of otitis media were present before the acquired animal is mixed with the rest of the herd. Even further, the farmer should partition the farm into various sections that can limit the spread of the disease in case of an outbreak. Continuous monitoring of the livestock is vital since it enables the farmer to identify the disease early on, which is essential in managing the spread of the disease. Pre-vaccination of calves against the viral pathogens before entering the feedlot, or vaccination upon arrival at the feedlot should be carried out to reduce morbidity by BRD (Howard et al., 1987; Nicholas and Ayling, 2003; Timsit et al., 2012)

Currently, there are few commercial and autogenous vaccines available and these are not effective in limiting the spread of *M. bovis* disease in animals. One of the challenges for vaccine

development is the lack of a consistent, reproducible experimental infection model for respiratory disease vaccine testing, the understanding of the role of other pathogens in the disease manifestation, the age of the animals involved, and the challenge dose used (Perez-Casal et al., 2017). Contradicting reports of successful disease models are available with studies using *M. bovis* only (Nicholas et al., 2002) and co-infection models using BHV-1 (Prysliak et al., 2011) during the challenge. Prysliak *et al*., also reported no disease after co-infection with BVDV-2 and *M. bovis* only infected animals (Prysliak et al., 2011). The discrepancies in the disease progression by *M. bovis* alone may be attributed to the age of the calves used in the respective studies.

We have reviewed the status of different vaccines used in *M. bovis* control (Perez-Casal et al., 2017). In dairy cattle, mastitis infected cattle vaccinated with a *M. bovis* bacterin did not confer any protection despite a high IgG1 and IgG2 titres response in the serum and they also observed no milk or circulating lymphocytes proliferation after infection (Boothby et al., 1986, 1987; Boothby et al., 1988). In the US there are only two licensed vaccines for the prevention of *M. bovis* infection (Soehnlen et al., 2011) in BRD. In an experimental vaccine trial a saponin-inactivated vaccine was shown to be safe and protective as when given to young calves on arrival to the farm reduced cost treatment and mortality. (Nicholas et al., 2002) In another study, a saponin inactivated *M. bovis* with Emulsigen^{TM} induced protection (Dudek et al., 2016). Use of live attenuated vaccines has also been explored with a multiple passaged Chinese *M. bovis* strain HB0101 that reduced the lung lesion scores (Zhang et al., 2014) but this is commercialised only in China. Commercially available live-attenuated vaccines in bovine, poultry, and swine have been used in control of mycoplasmosis in other countries other than those in Europe and North America (Perez-Casal et al., 2017).

In *M. mycoides subsp. mycoides* the current vaccine strategies include a killed vaccine that has poor efficacy and live vaccine that has issues with stability and safety (Mbulu et al., 2004) and only protects for 6-12 months (Wesonga and Thiaucourt, 2000). The live vaccines approved by FAO include 2 freeze-dried vaccines (T1SR and T1/44), (Thiaucourt et al., 2004). Hence the need for an improved vaccine that is affordable, stable, and easy to produce and provides better immunity, such as a subunit vaccine. This can be aided by reverse vaccinology (Rappuoli, 2000; Vivona et al., 2008). Using two *Mmm* genome sequences of strains PG1 (Westberg et al., 2004)

and Gladysdale (Wise et al., 2011) we identified several possible vaccine candidates (Perez-Casal et al., 2015) using reverse vaccinology. The potential surface exposed protein vaccine candidates were used to study their capacity to induce protection against an experimental CBPP infection. In the study, there was 80% reduction in lung pathology score and a correlation of high antibody titres to low pathology score index in two groups was reported. The antibodies may be playing a role in steric hindrance of *Mmm* attachment hence high titres of antibodies is a requisite for good immunity which explains why the live vaccine is short lived with decline in antibody titres (Nkando et al., 2016). In *M. bovis* multiples studies with protein vaccine candidates show no protection; in previous studies using recombinant proteins (Prysliak et al., 2013; van der Merwe et al., 2011) and membrane proteins (Mulongo et al., 2013). Further studies using immunoproteomics analysis and reverse vaccinology should be used to determine potential protective antigens as vaccine candidates and comparative genomics can be used to identify the ones conserved in all *M. bovis* isolates.

1.3. Bovine respiratory disease complex

Introduction

The bovine respiratory disease (BRD) complex can be described as a disease that affects the lower or upper respiratory tract of beef cattle. BRD is a multifactorial disease in which bacteria such as *Mannheimia haemolytica*, *M. bovis*, *Histophilus somni,* and *Pasteurella multocida;* and sometimes parasites like lung worms (Cusack et al., 2003) co-infect with viruses such as bovine herpes virus (BHV), parainfluenza virus (PI-3), bovine respiratory syncytial virus (BRSV), and bovine viral diarrhea virus (BVDV). The consequences of the viral infections are to compromise the immune system allowing the normal bacterial flora of the respiratory tract to cause secondary infections (Booker et al., 2008; Booker et al., 1999). Environmental factors such as transportation and housing of feedlot cattle also play a key role in the development of BRD and host factors such as the age of the cattle, immune status and previous health history of the animals cannot be ignored (Taylor et al., 2010). The treatment and control for BRD includes vaccination of viruses and the bacteria, antibiotics, and stress management during housing and transportation for control (Perez-Casal et al., 2017). BRD is known to resist antimicrobial medication, which poses a challenge in the treatment or management of the disease in animals and requires stronger antibiotics to mitigate (DeDonder and Apley, 2015).

1.4. Macrophages

Functions

Phagocytic cells such as macrophages or monocytes are important lines of defense against mycoplasmas in the respiratory tract. They play a crucial role in the orchestration and execution of the innate and adaptive arms of the immune response to bacterial infection. Macrophage effector functions are: phagocytosis mediated by a receptor, antigen presentation to other immune cells and production of cytokines (Gordon, 2007). Alveolar macrophages in particular, are important in the early clearance of mycoplasmas from the lungs. Previous evidence suggests a role of *M. bovis* in modulating alveolar macrophages activities although the mechanisms employed by *M. bovis* are not well understood (Maunsell et al., 2011).

Macrophage activation

In response to distinct external signals macrophages differentiate into three populations: classically activated, alternatively activated and regulatory macrophages. Upon polarization, macrophages differentiate in terms of cytokine production, surface receptor expression, effector function and chemokine repertoires and acquire different functional properties; host defense, wound healing and immune regulation respectively (Mosser and Edwards, 2008). The scheme in figure 1.1 shows the canonical macrophage activation pathways.

Classically activated macrophages are formed during cell-mediated immune responses. They produce pro-inflammatory cytokines (IL-1, IL-6, and IL-23) that are important components of host defense but can also cause extensive host tissue damage (Mosser and Edwards, 2008) as well as up-regulate major histocompatibility complex (MHC)-II surface receptor most of which can be detected by fluorescence-activated cell sorting (FACS) or qPCR assays. They protect the host from intracellular bacterial and viral infections. A combination of two signals is needed to produce classically activated macrophages *in vitro;* a primer IFN-γ produced by natural killer (NK) cells and T_H1 cells and interaction with Toll-like receptors ligands that induce production of TNFα, which in return stimulates macrophages. Classically activated macrophages are the only macrophage class able to metabolize arginine into NO for microbial killing by expressing inducible inducible nitric oxide synthase (iNOS) enzyme. In addition to iNOS activity, classically activated macrophages are associated with a shift towards increased glucose uptake (O'Neill *et*

al., 2013). Thus, by determining iNOS activity, increased glucose uptake, production of cytokines, and expression of the MHC-II receptor, we can identify classically activated macrophages.

Macrophages activated by the alternate pathway are associated with a T_H-2 type immune response and the indicator cytokine involved is IL-4 (Gordon, 2003; Stein et al., 1992). This phenotype produces less toxic oxygen and nitrogen radicals and is deficient in killing intracellular pathogens. Hölscher and his group suggested that they are more susceptible to intracellular infections (Holscher et al., 2006). Alternatively activated macrophages are characterized by an alteration in their metabolism of arginine and a reduction in the uptake of glucose (O'Neill and Hardie, 2013). Arginine is converted to ornithine and proline, a collagen precursor by the arginase I enzyme whose activity is induced by IL-4. Thus, measuring the increase of macrophage arginase I activity in the presence of IL-4, combined with glucose uptake assays will allow the identification of alternatively activated macrophages.

Even though in different studies regulatory macrophages have been developed using different methods *in vitro,* in all cases they secrete high levels of IL-10. They are also known to down-regulate the production of IL-12 as they shut off the production of the p40 subunit of IL-12/23. Currently, there is no proven reliable surface or biological markers hence the ratio of IL-10 to IL-12 and IL-23 cytokines could be used to define regulatory macrophages using an ELISA (Mosser, 2003; Sica et al., 2008).

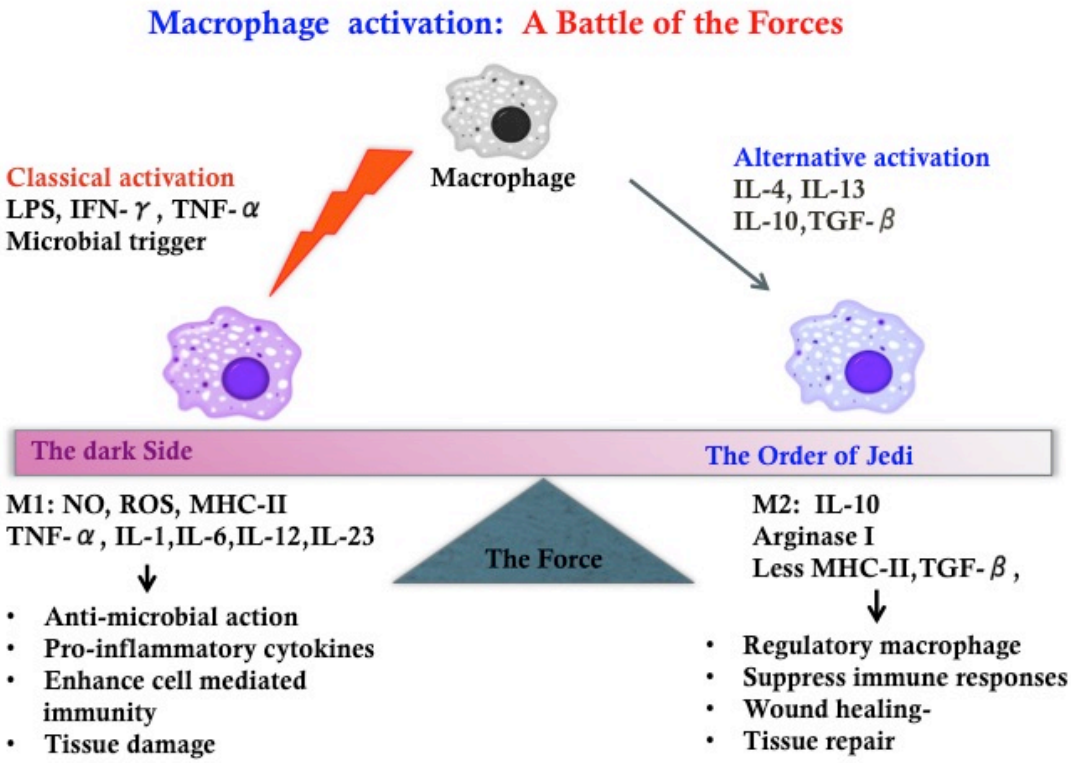


Figure 1. 1: Macrophage activation. Likened to the Star Wars movies and the battle between the forces, macrophages are activated to a classically activated phenotype (M1) and like soldiers of the Dark Side of the force releases pro-inflammatory mediators, cytotoxicity and contribute to pathogen clearance and if left unchecked, lead to tissue damage. The alternatively activated (M2) macrophages activities like the soldiers of the Order of Jedi counter the M1 activities (dark side) and dampen the immune responses and produce mediators involved in wound healing. Disease outcome depends on the balance between these 2 opposing forces. Adapted from (Laskin, 2009).

1.5. Use of cells lines as an approach to study host-pathogen interactions

The intracellular phase of *M. bovis* represents a protective niche for avoiding clearance by host immune cells as demonstrated by Kleinschmidt *et al*., in alveolar macrophages (Kleinschmidt et al., 2013). Host-pathogen interactions have been investigated over the past decades using a wide range of primary cell-dependent and -independent techniques using immortalized cell lines. A cell line that has been validated in experimental studies as models for *in vitro* survival, growth and as a co-infection model with other pathogens is needed. The dilemma for investigators to choose an *in vitro* cell model of either primary cells and/or cell lines is real as both are useful and have advantages and disadvantages.

Primary cells closely resemble the tissue from which they are derived (hence a better choice) but are isolated from different individual animals hence significant variability between samples is commonly encountered. The cells are often difficult to obtain, their isolation is time consuming and they are often poorly characterized. In contrast, immortalized cell lines are better characterized, show less variation, and are easy to grow (MacDonald, 1990).

The bovine macrophage cell line (BoMac) is an *in vitro* differentiated bovine peritoneal macrophage cell line (Stabel and Stabel, 1995). In particular, the development of the BoMac cell line has greatly facilitated the investigation of host-pathogen interactions as proved by an array of laboratory procedures in studies of *M. bovis* host-pathogen interaction. It has also been used to study BHV-4 (Donofrio and van Santen, 2001) and *Map* (Langelaar et al., 2005; Souza et al., 2007; Woo et al., 2006). In these different studies the cell line has shown the ability to develop macrophage functions including the ability to phagocytize bacteria with and without opsonization, generate ROS, produce different cytokines (IL6, TGFβ-1, TNFα-2, IFN-γ, IL1-α), and express genes related to apoptosis, the B-cell lymphoma 2 (BCL2-1) following activation and infection (Stabel and Stabel, 1995; Tooker et al., 2002). In a different study, Abendano and group showed an increased level of expression of the apoptotic inhibitor BCL2-1 and increased TGF-β after infection with *Map* (Abendano et al., 2013). In *M. bovis,* BoMac cells were used to test their potential use as an *in vitro* infection model for *M. bovis* with BVDV to be used in the future for synergism studies of *M. bovis* with other microorganisms (Burgi et al., 2018). *M. bovis*-host cell interactions seem to be dependent on the cell type, but the immune modulation of macrophage effector function in bovine mycoplasmosis by *M. bovis* is largely unknown. In this thesis work, I demonstrate the potential of BoMac cells for studies involving the mechanism of cell death after infection with *M. bovis*.

1.6. Cell death mechanisms

Introduction

Regulated cell death is essential in maintaining multicellular organisms during embryonic development, normal cell turnover in healthy adult tissue, immune responses, and clearance of infected cells (Tait et al., 2014). Any defect in cell death (too little or too much) contributes to different pathologies including cancer, autoimmunity, injury, and inflammation (Hotchkiss et al.,

2009). There are different highly regulated cell death mechanisms that are recognized for their morphologically and specific molecular differences and can be induced passively or actively by the cell itself (Green, 2011; Green and Llambi, 2015; Green and Victor, 2012). According to Galluzzi *et al*., the field continually expands; researchers unveil novel cell death mechanisms and pathways. Still, the fundamentals of cell death classification remain unchanged, encompassing the following types: necrosis, pyroptosis, necroptosis, autophagy, and apoptosis (Galluzzi et al., 2018).

Different cell death mechanism

Necrosis is referred to as the premature cell death through autolysis due to injury or response to lack of blood supply, infections, chemicals, and others (Banoth and Sutterwala, 2017). It is induced by external factors such as toxins, infections or unregulated cell digestion (Chang et al., 2017), but it does not however inform how the cell died (Schwartz and Bennett, 1995). It is characterised by high levels of cytosol Ca^{2+} accumulation, activation of calcium-activated proteases (calpains), and phospholipases (Fawthrop et al., 1991; Furuse et al., 2015; Vanden Berghe et al., 2014), chromatin condensation and DNA fragmentation is lacking (Edinger and Thompson, 2004).

Necroptosis, on the other hand, is a genetically regulated necrosis and a defence mechanism that helps the cell to execute suicide in the presence of an inhibitor of cysteinyl aspartic acid-protease (caspase) in a caspase-independent way (Pasparakis and Vandenabeele, 2015). It is induced by stimuli such as radiation, pathological or endogenous factors, chemical or viral exposure and others (Legarda *et al.* 2016). It is dependent on death receptor ligands such as TNF and fatty acid synthetase (Fas) ligands (Matsumura et al., 2000), and also through pattern recognition receptors (PRRs) such as TLRs (Galluzzi et al., 2017; Gonzalez-Juarbe et al., 2017). Necroptosis requires sequential activation of receptor-interacting serine/threonine-protein kinases, RIPK1 and RIPK3, and its substrate mixed lineage kinase domain (MLKL). It is characterized by swelling of organelles (Moujalled *et al.* 2014), and once phosphorylated, the pMLKL disrupts the cellular membrane that leads to random degradation of DNA, acidosis, along with leakage of cellular content leading to a programmed cell death (PCD) (Lalaoui *et al.* 2015).

Pyroptosis is referred to as an inherent inflammatory form of PCD, which frequently occurs when infection happens with intracellular pathogens and is likely to take part in the antimicrobial

response (Doitsh *et al.* 2014). Pyroptosis resembles apoptosis in cell lysis, swelling, pore formation, and DNA fragmentation (Shi *et al.* 2017) but is dependent on inflammatory caspases; caspase 1 (Cookson and Brennan, 2001), caspase 3, murine caspase 11 (Case et al., 2013; Kayagaki et al., 2011), and its human homologs caspases 4 and 5 (Aachoui et al., 2013; Fernandez and Lamkanfi, 2015). The inflammatory caspases induce the cleavage of members of the gasdermin D protein family, releasing the gasdermin-N domain that is often involved in the formation of plasma membrane pores that lead to leaking of cellular content that causes inflammation (Ruhl and Broz, 2016; Shi et al., 2017; Wang et al., 2017). Pyroptosis is often (if not always) followed by IL-1β and IL18 secretion, and hence mediates robust pro-inflammatory effects and fever (Galluzzi et al., 2018). The changes in cell death by pyroptosis include the release of alarmins and DAMPs (Jorgensen and Miao, 2015).

Autophagy is referred to as the natural physiological process which involves cell destruction in the host that is done to maintain homeostasis or normal protein degradation; clearance of defective organelles (Galluzzi et al., 2017), and the recycling of new information from destroyed organelles. (Diaz et al., 2005). Autophagy of the cells is induced by hypoxia, starvation, growth factor deprivation, and chemotherapy. The observable changes in the autophagy cells include mass degradation of proteins and organelles along with double membrane vacuolization in the cytosol, autophagosomes (Su *et al.* 2015). The molecular changes in the cells include lysosomal activity, fusion of autophagosomes with the lysosome (Galluzzi et al., 2017). During clearance, the cells are cannibalized, and the contents present in the cell are recycled to survive the tissues (Zhuang et al., 2016).

Apoptosis

Apoptosis, “controlled suicide’, is a programmed cell death which occurs in all multicellular organisms during embryonic development, regeneration of healthy cells in adult tissue, and cellular stress-related signals including the presence of toxins and withdrawal of hormones (Kerr et al., 1972). As mentioned by Renehan *et al.*, cells that undergo apoptosis are seen to shrink and developed bubble-like projections on the cell surface. The DNA present in the nucleus of the apoptotic cell is fragmented into smaller pieces along with fragmentation of the cellular organelles. The cell at the end then splits into smaller apoptotic bodies in the form of cellular debris, which are engulfed by the macrophages but with no inflammatory response (Fulda et al.,

2010; Hotchkiss et al., 2009; Renehan et al., 2001). As postulated by Hochreiter-Hufford *et al.*, apoptosis leads to the clearance of damaged, cancerous or infected cells. This helps in protecting the body from infections and disease and can also be used by the pathogen to evade the host immune response (Hochreiter-Hufford and Ravichandran, 2013). Apoptosis is also seen to play a key part in the maintenance and development of an effective immune system. This is because when T- and B-cells are produced they are then put to the test if they act against their body's own defence system. The cells that act against the defence system are instantly eliminated through the help of apoptosis (Mariño *et al.* 2014). Moreover, during detection of a pathogen in the body huge numbers of pathogen-specific immune cells are released into the body for the destruction of the pathogeṇ. However, after the pathogen is removed the pathogen-specific immune cells are removed by apoptosis to help the body maintain homeostasis (Hochreiter-Hufford and Ravichandran, 2013). There are two major apoptosis pathways; the intrinsic pathway also referred to as the mitochondrial pathway; is induced by growth factors, radiation, toxins, and hypoxia (Wang and Youle, 2009), while the extrinsic pathways also known as the death receptor pathway is activated by transmembrane receptor-mediated interactions like activation of TNF (Teng and Hardwick, 2014). Both pathways result in activation of caspases (Duprez et al., 2009; Teng and Hardwick, 2014) and can be blocked by inhibitor of apoptosis proteins (IAPs) allowing for cell survival (Elmore, 2007; Riedl and Shi, 2004; Shi, 2002).

Intrinsic pathway

The intrinsic pathway is initiated by non-receptor induced stimuli such as during radiotherapy or chemotherapy. Thus, it is activated when a range of endogenous as well as exogenous stimuli like DNA damage, oxidative stress, ischemia are experienced by the host and these leads to the loss of mitochondrial membrane potential via opening of the mitochondrial permeability transition pore (Joo *et al.* 2015). Reactive oxygen species (ROS) also play a significant role as a stimulus for the intrinsic pathway. Once the mitochondrial membrane integrity is lost it leads to leakage of sequestered pro-apoptotic proteins cytochrome *C* (Saelens et al., 2004) and second mitochondrial activator of caspases/direct (Smac/DIABLO) protein, (Du et al., 2000) into the cytosol. Once in the cytosol cytochrome *C* binds to apoptotic protease activating factor (Apaf-1) and procaspase 9 to form an apoptosome. The complex formed is seen to hydrolyse Adenosine triphosphate (ATP) for cleavage and activation of caspase 9. The initiator caspase 9 is then involved in activating and

cleavage of the executioner caspases 3, 6, and 7 that eventually lead the cell to undergo apoptosis (Park *et al.* 2016). The intrinsic pathway activation also includes activation of Bcl-2 homologs (Redza-Dutordoir and Averill-Bates, 2016; Wang and Youle, 2009; Wang, 2001) that can either promote or inhibit apoptosis. This family of proteins includes the anti-apoptotic Bcl-2-family proteins such as BCL2 related protein long isoform (Bcl-X_L), myeloid leukemia cell differentiation protein (Mcl-1), and Bcl-2), and pro-apoptotic family of proteins such as BCL2 associated X protein, Bax and BCL2 antagonist of cell death, Bad (Elmore, 2007; Riedl and Shi, 2004; Shi, 2002). The pro-apoptotic Bcl-2 family proteins are found in the cytosol (Yee *et al.* 2014) and the anti-apoptotic proteins are localized in the outer mitochondrial membrane as heterodimers with apoptotic proteins (Bax and Bad) thus inhibiting their apoptotic functions (Cory and Adams, 2002) or by controlling the cleavage and activation of the caspases (Czabotar *et al.* 2014). Smac/DIABLO pro-apoptotic proteins released in the cytosol are thought to also induce apoptosis by interacting with the inhibitor of apoptosis proteins to inhibit their activity (Wu et al., 2000). IAPs are a structural and functional family of proteins which execute their function as negative regulators of apoptosis and caspases (Silke and Meier, 2013). All the IAPs have a common feature that is the presence of one or more copies of the baculovirus IAP repeat motifs (BIR) (Birnbaum et al., 1994). The BIR found in IAPs bind to IAP-binding motifs (IBMs) present in caspases inhibiting their protease activity (Riedl and Shi, 2004), and Smac/DIABLO an IAP-antagonist, inhibiting apoptosis (Wu et al., 2000). The IAPs that are usually found in mammals include XIAP, cIAP1, and cIAP2, NAIP, Survivin, and others (Srinivasula and Ashwell, 2008). The third group of pro-apoptotic proteins released from the mitochondria includes endonuclease G that occurs in the late stage of apoptosis when the cell has already committed to PCD. The endonuclease translocates to the nucleus where it causes DNA fragmentation (Li et al., 2001).

Extrinsic pathway

The extrinsic pathway is stimulated by external death-induced signals for apoptosis and each of the cells possesses receptors in the plasma membrane which are specific for each stimulus and therefore the extrinsic pathway is also called the receptor-mediated programmed cell death pathway. In most cases, tumor necrosis factor alpha (TNF-α) is the cytokine that is responsible for the external stimuli in initiating the extrinsic pathway of apoptosis. TNF is an inflammatory

cytokine mostly provoked in the body when the body is exposed to toxic substances and infection (Teng and Hardwick, 2014). The extrinsic pathway of apoptosis involves binding of the TNF to the tumor necrosis factor receptor-1 (TNFR-1) on the plasma membrane of the cell (Locksley et al., 2001). The TNFR-1 belongs to a member of the death receptor family that activates the apoptotic signal process. The TNFR-1 is a transmembrane receptor which has the external domain for bonding of ligand along with a cytosol domain (Nair et al., 2014), the cytosolic domain present in each subunit of TNFR-1 contains a 70 amino acid segment known as the death domain (Chattopadhyay *et al.* 2014). The binding of the TNF to the TNFR-1 causes a change in the death domain, which results to recruit many apoptotic-related adaptor proteins factors. To further activate the death domain, the cytosolic adapter proteins; TNF receptor-associated death domain (TRADD) and Fas-associated death domain (Rahman and McFadden) along with procaspase 8 residue are bound in forming a multi-protein, death-inducing signalling complex (DISC) which interacts in generating an activated caspase 8 (Elmore, 2007). This activated caspase 8 acts as an initiator in activation of the extrinsic pathway of apoptosis which in turn activates caspase 3 which is the key apoptotic executioner creating self-destruction of the cell (Richardson *et al.* 2017). The Fas-ligand is also seen to cause extrinsic apoptosis by activating procaspase 8, which in turn activates the downstream executioner caspase 3 for inducing apoptosis (Ahmed *et al.* 2015).

Caspases

Caspases belong to enzymes of the cysteine protease family which have an essential role in executing programmed cell death and inflammation (Kumar, 2007). The caspases are divided into three groups that include initiator caspases (caspases 8, 2, 9 and 10), executioner caspases (caspases 7, 3, and 6), and inflammatory caspases (caspases 1, 4, 5, 11 and 12) (Cohen, 1997; Fernandez and Lamkanfi, 2015). The initiator caspases act to initiate the apoptotic process whereas the executioner caspases execute the proteolysis that leads to the cleavage of cellular proteins. The caspases are initially synthesized in the form of inactive procaspases which are later cleaved as well as activated to respond to death receptors, granzyme B, and apoptosome stimuli depending on the stimuli (McIlwain et al., 2013). The activated caspases then go to cleave various substrates that include nuclear proteins, downstream caspases, and plasma membrane proteins that lead ultimately to execute cell death (Slee et al., 2001).

Nuclear Factor kappa beta (NF-kβ) pathway role in apoptosis

NF-kβ is a form of transcription factor composed of c-Rel, RelA, RelB, p105/NF-kβ1 and p100/ NF-kβ2 proteins, that are involved in different pathways including immune and inflammatory responses (Hayden et al., 2006). The canonical NF-kβ pathway that plays a role in apoptosis is activated by cellular stress either from within the cell, the environment, toxins, medications or infections. Usually, RelA/p65 is stored in an inactive form in the cytoplasm as part of the p65-p50 (NF-κβ) complex bound to the inhibitory protein IκBα. On activation, IκBα is phosphorylated by the IκB kinase leading to ubiquitination and release of the inhibitory protein from the NF-kβ complex (Scheidereit, 2006). This in turn, exposes nuclear import factors that target p65 to the nucleus where it binds to regulatory elements and leads to changes in cellular gene transcription (Rahman and McFadden, 2011). Upon activation, NF-κβ leads to expression of apoptotic or anti-apoptotic genes of the Bcl-2 family, the IAPs, and the IAP antagonists (Into et al., 2004a; Kucharczak et al., 2003).

Apoptosis programmed cell death (PCD) and infection

While apoptosis is considered a defence mechanism for the host during pathogen invasion, by clearance of infected cells without inflammation, bacterial pathogens have also evolved strategies to manipulate host cell death mechanisms for their survival, multiplication as well as dissemination (Renehan et al., 2001). Using different virulence factors, pathogenic bacteria are known to either inhibit apoptosis of the host cells to allow proliferation and dissemination or induce apoptosis to eliminate the different types of immune cells.

Mycobacteria are intracellular pathogens that are taken up through receptor-mediated phagocytosis by macrophages and modulates their effector functions; their virulence largely depends on intracellular survival in the macrophages (Basu et al., 2012). Virulent *Mycobacteria* are known to use either of the two key apoptotic pathways and possess both apoptotic inducing and inhibiting components (Briken and Miller, 2008). In *Mycobacterium avium* the MAV2052 protein is known to induce apoptosis through TLR-4 in murine macrophages through the intrinsic pathway activated by the production of ROS that leads to loss of mitochondrial membrane potential ($\Delta\Psi_m$), the release of pro-apoptotic protein Bax and cytochrome *C* and activation of caspases (Lee et al., 2016). In *Mycobacterium tuberculosis* (Mtb), other proteins have been

implicated in the induction of TLR-2-dependent activation that results in activation of the NF-kβ pathway and release of pro-apoptotic activity of TNF-α (Basu et al., 2007). Moreover, a glycolipid component released from the mycobacterial wall, lipoarabinomannan (LAM) induce apoptosis (Guerardel et al., 2003) or inhibit apoptosis through phosphorylation of Bad, which prevents the binding with the anti-apoptotic proteins Bcl-xl and Bcl-2 (Halder et al., 2015; Maiti et al., 2001) or by neutralising the pro-apoptotic activity of TNF-α (Srinivasan *et al.* 2014).

Neisseria gonorrhoeae induces apoptosis by releasing a membrane protein porin (PorB) associated with loss of membrane potential, the release of cytochrome *C,* and activation of caspases (Deo *et al.* 2018). Moreover, *Neisseria meningitidis* possesses lipopolysaccharide (LPS) and LPO that induce apoptosis in epithelial cells (Deghmane et al., 2009) and the porin PorB that inhibits apoptosis in epithelial and lymphoid cell lines (Massari et al., 2000). Similarly, *Salmonella spp*. directly injects effector SopB through a type III secretion system resulting in activation of the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) pathway. The pathway reacts to prevent the release of cytochrome *C* resulting in inhibition of apoptosis (Robinson and Aw, 2016). While in *Anaplasma phagocytophilum* the PI3/Akt pathway is activated along with activation of the NF-kβ pathway and are also seen to prevent the release of cytochrome *C* along with activation of the IAPs (Ayllón *et al.* 2015) and secretion of Ats-1 protein to block pro-apoptotic Bax to inhibit apoptosis (Niu et al., 2010).

As discussed, different pathogenic bacteria modulate apoptosis by either, protecting the mitochondria integrity preventing release of cytochrome *C* and caspase activation as described in *Chlamydia* and *Neisseria spp.* (Greene *et al*., 2006), or activating cell survival pathways by up-regulating inhibitors of apoptosis (e.g. Bcl-x proteins) as described for *Rickettsia rickettsii* (Lancellotti *et al*., 2006). The discrepancies of the same bacteria possessing ability to induce or inhibit apoptosis are attributed to the different cell types, different strains, and the different antigens/virulence factors used in the studies. The overlapping effects of both induction and inhibition of apoptosis are likely to play a role in the pathogenesis of the disease. Like other pathogens, *M. bovis* has evolved to use apoptosis to modulate the immune system of the host as shown by Mulongo *et al*., using high-throughput bovine peptide kinome arrays as one of the pathways modulated during infection of bovine monocytes (Mulongo et al., 2014). We have shown that *M. bovis* strain Mb1 delays apoptosis in bovine monocytes, PBMCs, and primary

alveolar macrophages for survival (Van der Merwe *et al*., 2010; Mulongo *et al*., 2014; Suleman *et al.,* 2016); and induces apoptosis in bovine neutrophils to enhance release of bacteria for colonization of new cells to assist in dissemination (Jimbo et al., 2017). In another study with lymphocytes, Vanden Bush reported that *M. bovis* induced apoptosis after observing lymphotoxicity post-*M. bovis* infection (Vanden Bush and Rosenbusch, 2002) while in the BoMac cell line, the *M. bovis* strain JF4278 caused slight apoptosis and delay in STS-induced apoptosis was not observed. These discrepancies may also reflect the diversity that exists within *M. bovis* strains as previously described in adherence to various host cell lines that influence virulence (Burgi et al., 2018; Thomas et al., 2003a). Moreover, Wiggins' research group demonstrated that the ability to reduce ROS and cellular metabolism in leucocytes varies within different strains therefore suggesting, "research that examines the effect of a single *M. bovis* isolate may not yield information broadly relevant to all *M. bovis* isolates" (Wiggins et al., 2011).

In *Mycobacterium spp.* after two decades of research, there are many studies that associate different virulence genes/antigens as the drivers of apoptosis inhibition in macrophages (Parandhaman and Narayanan, 2014). In *M. bovis* no genes have been identified responsible for apoptosis and this highlights the large knowledge gap in the field of mycoplasmas. In Mtb*, Mycobacterium bovis* and *Mycobacterium kansasii*, it has been suggested that only virulent strains inhibit apoptosis in macrophages as attenuated strains like the BCG-strain enhanced apoptosis (Keane et al., 2000). This has led to several studies where researchers knocked-out different virulence genes and reported an increase in macrophage apoptosis (Velmurugan et al., 2007). Hence, if the genetic formation of a bacterium influences the mechanism of apoptosis then, this would make a huge contribution in creating attenuated vaccine strains. Such key information is missing in the *M. bovis*-host interaction field and there is a need for further investigations to find out if both pathogenic and non-pathogenic strains of *M. bovis* inhibit apoptosis of primary alveolar macrophages. From a basic research point of view, the discovery of genes responsible for apoptosis provides an opportunity to understand the host-pathogen interaction further and inform the pathogenesis of the disease. In this thesis, I will explore the molecular mechanisms employed by *M. bovis* to modulate macrophage apoptosis as currently there is limited understanding of the strategies used.

HYPOTHESIS

Immune modulation by *Mycoplasma bovis* and its interaction with host cells continues to be investigated to generate insights into disease pathogenesis outcomes. Mycoplasmas interact with host immune cells to create a favourable niche for their replication and thus, avoid clearance. To understand disease progression and how to combat it, it is necessary to study mycoplasma-host interactions. I hypothesize that *Mycoplasma bovis* strain Mb1 invades and modulates bovine alveolar macrophage (BAM) activity and apoptosis, while avoiding clearance by the host immune system.

OBJECTIVES

1. Characterize the heterogeneity of activated *M. bovis* infected primary bovine alveolar macrophages based on their cytokine and nitric oxide production.
2. Determine the antigen-presentation capacity of primary BAMs infected by *M. bovis in vivo* using ova-albumin and characterise the immune responses.
3. Determine the mechanism of BoMac cell apoptosis induced by the cattle isolate *M. bovis* strain Mb1 and the bison isolate strain Mb304.
4. Study the role of the NF-κβ-signalling pathway on apoptosis of BoMac cells infected with *M. bovis* strains Mb1 or Mb304 by detecting the translocation of the p65 subunit of NF-κβ to the nucleus and the differential gene expression of anti- and pro-apoptotic genes.

CHAPTER 2. Understanding the heterogeneity of responses in primary bovine alveolar macrophages activated by the *M. bovis* strain Mb1

2.1. Introduction

The importance and prevalence of *M. bovis* among other pathological agents implicated in bovine respiratory disease (BRD) complex, a multifactorial disease of feedlot cattle, has been shown (Arcangioli et al., 2008). It is also the most pathogenic bovine mycoplasma in Europe and North America causing substantial economic losses (Maunsell et al., 2011). A significant line of defense against mycoplasmas in the respiratory tract is macrophages. They are responsible for initiating both the innate and adaptive arm of the immune response to bacterial infection. Pathogens are known to exploit the different polarized phenotypes of macrophages to the disadvantage of the host (MacMicking et al., 1997a; Odegaard and Chawla, 2011). A study on experimental challenge models for *M. bovis* in feedlot cattle indicate that *M. bovis* is capable of persisting in lungs of recovered animals (Prysliak et al., 2011; Rodriguez et al., 2015). Naturally occurring *M. bovis* persist in a herd over an extended period and can be consistently identified not only in lesions but also commonly in healthy lungs and those with pneumonia (Gagea et al., 2006b). This suggests that *M. bovis* can evade alveolar macrophage functions although the mechanisms employed are not well understood. Hence, I sought to investigate the phenotype of BAMs activated in response to an *in vitro* infection with the *M. bovis* strain Mb1. Alveolar macrophages were isolated and infected with Mb1. The supernatant and cells were harvested at different time points post-infection and assayed for nitric oxide production, cytokine profile and expression of surface markers associated with APCs. I observed a reduction in the production of nitric oxide and the pro-inflammatory cytokine TNF-α coupled with a reduction in gene transcription of iNOS, the nitric oxide synthase gene. These results suggest modulation of the immune response by the *M. bovis* skewed to an anti-inflammatory immune response.

2.2. Materials and Methods:

Bacteria strains and culture conditions

The experiments were conducted using *M. bovis* strain Mb1, which was previously isolated from the synovial fluid of a calf exhibiting signs of arthritis (Perez-Casal and Prysliak, 2007). Cultures were grown in modified Hayflick's medium at 37°C in a 5% CO_2 atmosphere. Bacterial cells

were collected by centrifugation (5,500 x g for 15min) at the exponential phase of growth and then washed with minimum essential medium (MEM; Invitrogen, Burlington, ON, Canada). Bacteria were suspended to a cell density of 10^8 cfu/ml in MEM supplemented with 30% glycerol and stored at -70°C until use.

Isolation of bovine alveolar macrophages (BAMs)

The lungs of recently euthanized animals were gradually filled via the trachea with sterile Hanks balanced salt solution (HBSS) supplemented with 2X antibiotic-antimycotic cocktail (penicillin, streptomycin, and amphotericin B) and 1X gentamicin. The lungs were gently massaged, and the lavage decanted into sterile bottles to harvest the BAMs suspension. Washing of the lungs required 3 to 4L of HBSS (dependent on the lung size) and about one-half of this volume could be recovered. The macrophage suspension was centrifuged at 400 × *g* for 10min at 4°C and the cell pellet was collected and washed with cold PBS supplemented with 2X antibiotic-antimycotic and suspended in Dulbecco's modified eagle medium (D-MEM). The cells were then counted in a Coulter counter and the suspension diluted to the required working concentration of BAMs. D-MEM containing 10% fetal bovine serum (FBS), 0.05 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids (NEAA), and 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer was used to culture BAMs for all subsequent assays. Trypan blue 0.4% and flow cytometry was used to assess the viability of the BAMs.

Infection of BAMs with *M. bovis*

Alveolar macrophages from individual animals were seeded (5 x 10^6/per well) in flat-bottom 6-well tissue culture plates (Nunc™, Thermo Fisher Scientific, Massachusetts, USA). The cells were incubated at 37°C in 5% CO_2 atmosphere for 3h. The supernatant was discarded and replaced with 2ml of fresh D-MEM medium and the cells were incubated overnight at 37°C in 5% CO_2 atmosphere. Infection with Mb1 was carried out using 2.5 x 10^7 cfu/ml of *M. bovis* at a multiplicity of infection (MOI) of 0.1:1 and 5:1. The cultures were incubated at 37°C in a 5% CO_2 atmosphere. The supernatants were collected at different time points (3h, 6h, and 24h) and assayed for the presence of the following cytokines: IL-1, IL-6, IL-10, IL-12, IL-13, IFN-γ, IFN-α, TNF-α, and TGF-β using a BioRad BioPlex® 200 reader (BioRad, CA, USA). Eight independent experiments with two technical replicates of each treatment were performed.

Bio-Plex® cytokine ELISA

Different colour-coded BioRad MaxPlex-® microsphere beads (BioRad, CA, USA) were coated with antibodies to the target cytokines. The cytokines coupled colour-coded beads (Appendix, table 6.1.) were added at 1200 beads/well at a final volume of 50µl/well, to a 96-well plate, and washed with PBS-BN (PBS, 1% bovine serum albumin (BSA), 0.05% Na azide, pH 7.4) using the Bio-Plex Pro™ Wash Station (BioRad, CA, USA). The cytokine standards, samples, and PBS-BN as a blank were added to the beads at 50µl/well and incubated at 750rpm at room temperature (RT) for 30min on a shaker (IKA MTS 2/4 digital microtiter shaker, NC, USA) followed by washing with PBS using an automatic plate washer. Detection of cytokines was performed using biotinylated antibodies at 50µl/well diluted in PBS-BN. The plates were incubated as above and washed with PBS before adding Strepatavidin-Phycoerythrin (SA-PE; Cedarlane, ON, Canada) at 5µg/ml in PBS-BN (50ul/well) and the plates were incubated at RT for 10min on the plate shaker at 750rpm. The plates were then washed with PBS. Finally, 125µl of TE buffer was added to each well and the plates incubated at 750rpm at RT for 3min before reading the cytokine concentrations on the Bio-Plex® at 45sec timeout, 50 beads/region, 100µl volume following the manufacturer's instructions. During all the shaking the plates were sealed and wrapped in foil to avoid light.

Nitric oxide (NO) concentration in BAMs supernatant

Nitric oxide concentration was determined by measuring nitrite levels in supernatants of macrophage cultures using a modification of the Griess reaction a colorimetric assay, (R&D Systems, Minneapolis, USA), that detects nitrite formed by the spontaneous oxidation of NO under physiological conditions. Alveolar macrophages isolated as before were seeded, (5 x 10^6/per well) in flat-bottom 6-well tissue culture plates (Nunc™, Thermo Fisher Scientific, Massachusetts, USA). The cells were incubated at 37°C in a 5% CO_2 atmosphere for 3h. The supernatant was discarded and replaced with 2.5ml of fresh D-MEM medium and incubated overnight at 37°C in 5% CO_2 atmosphere. Infection with Mb1 was carried out using 2.5 x 10^7 cfu/ml of *M. bovis* at a multiplicity of infection (MOI) of 5:1. The cultures were incubated at 37°C in a 5% CO_2 atmosphere and the supernatant collected at different time points (0h, 3h, 6h, 12h, and 24h) and assayed for the presence of nitrites. In a 96-well microplate (Nunc™, Thermo Fisher Scientific, Massachusetts, USA), 20µl of Griess reagent (equal volumes of *N*-(1-

naphthyl)ethylenediamine and sulfanilic acid) was added to 150µl of the nitrite-containing sample and 130µl water and incubated for 30min at RT. The absorbance of the nitrite-containing samples relative to the reference sample (Griess reagent and water), was measured at 548nm using a Bio-Rad Xmax spectrophotometric microplate reader. The absorbance readings were converted to nitrite concentrations using a standard curve of nitrite (NO_2^-) solutions with concentrations between 1-100µM. Eight independent experiments with two technical replicates of each treatment were performed.

Flow cytometry

Alveolar macrophages were isolated from three animals as indicated previously and seeded, (5 x 10^6/per well) in flat-bottom 6-well tissue culture plates (Nunc™, Thermo Fisher Scientific, Massachusetts, USA). The cells were incubated at 37°C in a 5% CO_2 atmosphere for 3h. The supernatants were discarded and replaced with 2ml of fresh D-MEM medium and plates incubated overnight at 37°C in 5% CO_2 atmosphere. Infection with Mb1 was carried out using 2.5 x 10^7 cfu/ml of *M. bovis* at a multiplicity of infection (MOI) of 5:1. The cultures were incubated at 37°C in 5% CO_2 atmosphere for 24h. The adherent cells were washed with PBSA and 1ml of pre-warmed Accutase™ (Sigma Inc, St. Louis, MO, USA) was added to cover the entire surface of the tissue culture and incubated for 30min to allow cells to detach. Cells were transferred into a centrifuge tube and collected by centrifugation at 400-x g for 5min at 4°C. The supernatants were discarded and the cell pellets saved for the assays. The cells were counted and blocked in PBSA with 2% BSA for 30min. Different fluorophore-conjugated antibodies were added to the sample in appropriate dilutions (Appendix, table 6.1) and stained for 15min in an orbital shaker in the dark followed by incubation with a secondary antibody where necessary, with washes in between. The fluorophore-conjugated antibodies used were, anti macrophage-APC, CD40-RPE, CD80-PE, CD163-PE, CD86-FITC, CD205-FITC, MHC II-None (secondary antibody IgG1-PE) and CD11b-FITC. Samples were stained with the secondary antibody only to control for non-specific binding. The cells were washed and suspended in FACS buffer (PBS with 2% FBS and 0.1% Na azide) for flow cytometry analysis. Samples were analysed with (BD FACSCalibur™, San Jose, CA) and data analysed with Kaluza® software (Beckman Coulter, Indianapolis, US). Minimum of 10,000 events were captured for each sample with data collected in list-mode and

cells gated according to size (FS: forward scatter) and granularity (SS: side scatter). PI was used to measure the viability of cells.

Quantitative real-time PCR

Total RNA was extracted using Trizol® (Molecular probes, Life technologies®, USA) from BAMs infected with *M. bovis*, cells treated with lipopolysaccharide (LPS) for 24h, and un-infected BAMs as control from three independent experiments. RNA quantity was determined using a Nanodrop spectrophotometer and integrity was assessed using denaturing agarose gel electrophoresis. Reverse transcription was performed using primers in Table 2.1; they all had a primer efficiency of above 90%. qPCR was carried out using the RT² SYBR Green Fluor (Qiagen, CA, USA) fast method performed on an iCycler i5 system (BioRad, CA, USA). The reactions were carried out in duplicate on 5µl of cDNA diluted tenfold with H_2O. The melting curve analysis indicated a single amplicon product for each primer pair. The data are expressed as fold changes as calculated using the $2^{-\Delta\Delta CT}$ method relative to GAPDH, a stable housekeeping gene.

Table 2. 1: Primers used in this study.

Gene name	Primer sequence	Product length (bp)	Reference
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	F: TTCAACGGCACAGTCAAGG R: ACATACTCAGCACCAGCATCAC	119	This thesis
Beta-actin (β-Actin)	F: AGGCATCCTGACCCTCAAGTA R: GCTCGTTGTAGAAGGTGTGGT	95	This thesis
Toll-like receptor 2 (TLR-2)	F: CTGTGTGCGTCTTCCTCAGA R: TCAGGGAGCAGAGTAACCAGA	113	This thesis

Toll-like receptor 6 (TLR-6)	F: CGACATTGAAGGCACTGAAA R: TCCTGAGGACAAAGCATGTG	110	This thesis
Ribosomal protein S15a (RPS15a)	F: GAATGGTGCGCATGAATGTC R: GACTTTGGAGCACGGCCTAA	101	(Kishore et al., 2013; Sodhi et al., 2013)
Ribosomal protein S9 (RPS9)	F: CCTCGACCAAGAGCTGAAG R: CCTCCAGACCTCACGTTTGTTC	54	(Kishore et al., 2013; Sodhi et al., 2013)
Inducible nitric oxide synthase enzyme (iNOS)	F: GGTGGAAGCAGTAACAAAGGA R: GACCTGATGTTGCCGTTGTTG	115	This thesis
Tyrosine 3-monooxygenase/ Tryptophan 5-monooxygenase activation protein, zeta polypeptide (YHZ)	F: GAGCTGGTACAGAAGGCCAAAC R: ATGACCTACGGGCTCCTACAAC	160	This thesis

The sequences of housekeeping and TLR genes primers used in the study. F: Forward primer and R: Reverse primer.

Statistical Analysis

Prism 7.0c (GraphPad Software, Mac OS X, La Jolla, CA, USA) was used for data analysis. I used one-way analysis of variance (ANOVA, Friedman or Kruskal-Wallis tests) to analyse the cytokines, nitric oxide production, surface receptors, and differential gene expression data followed by multiple-groups analysis to compare the different treatments. Differences were considered significant at a P value of ≤ 0.05.

2.3. Results

BAMs recovery and viability

BAL is widely accepted as a clinical sample for lower respiratory infections (Thomas et al., 2002) and the BAL cells composition includes alveolar macrophages, lymphocytes, eosinophils, epithelial cells and neutrophils. I sought to determine the percentage of BAMs recovered from the lavage and their viability before using them for different assays and after infection or treatment and the results are shown in figure 2.1. Following overnight culture and removal of non-adherent cells, the cell viability on the day of BAMs harvesting from BAL was greater than 96% of the adherent cells as assessed by trypan blue from each of the animals (not shown). The viability of the BAMs was also demonstrated by flow cytometry to be more than 93% even after 24h incubation with or without treatment (Fig. 2.1a). Cells were stained with propidium iodide (PI), a DNA binding dye used to check if the integrity of the membrane is compromised, the amount of PI detected is inversely proportional to the percentage of viable BAMs cells.

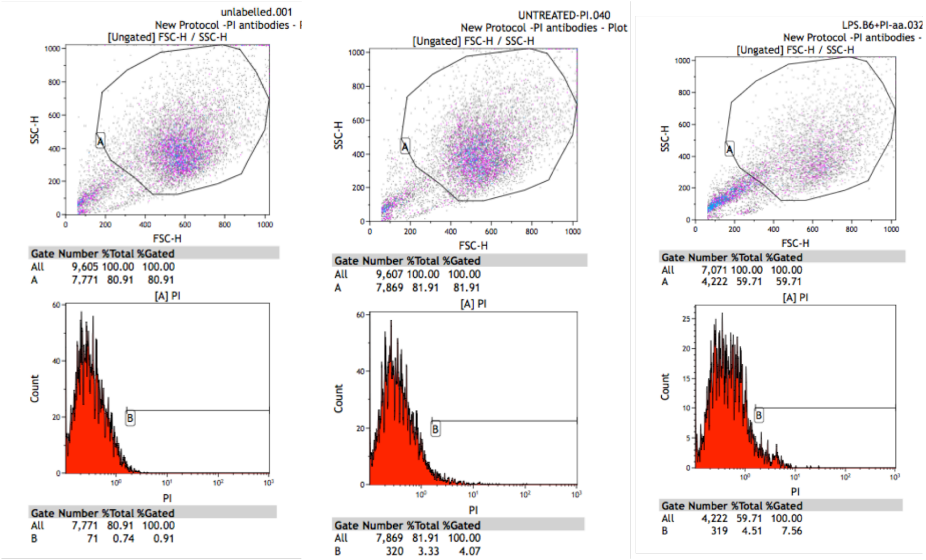


Figure 2. 1a: Representative plot of flow cytometry analysis demonstrating cell viability. Cell viability after 24h incubation of unlabelled BAMs, untreated BAMs and LPS-treated BAMs were all above 93% with the fluorescence dot graphs and the scatter box plots show PI positive (nonviable) cells.

To assess the population of BAM cells in BAL fluid, the cell suspension was stained with a mouse anti-human macrophage APC antibody (Serotec, CA, USA). The forward scatter height (FSC-H) vs. forward scatter area (FSC-A) (R1) and FSC-A vs. side scatter area (SSC-A) were used for gating on single cells to eliminate debris and clumped cells from the analysis. Single cells were then examined by CD expression, gating on anti-macrophage APC^{+} cells, which represent total alveolar macrophage present (Fig. 2.1b).

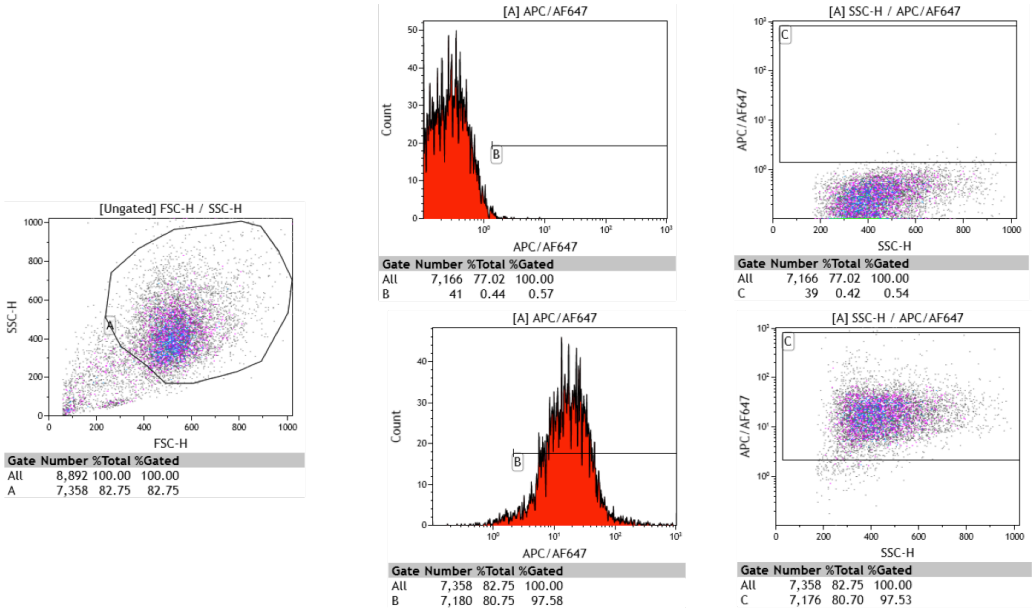


Figure 2.1. b: Representative plot of flow cytometry analysis demonstrating the live population of BAMs in lavage fluid. Single-cell suspension BAL cells were gated using the forward scatter and side scatter based on size and granularity respectively, then followed by showing the fluorescence peak shifting of the BAMs before and after staining with a mouse anti-macrophage APC marker.

The lung lavage fluid contained 98% of alveolar macrophage cells as shown by the anti-macrophage APC^{+} cells where there was a shift in intensity between the negative control and stained cells (Fig. 2.1b).

Lack of stimulation of BAMs TNF-α induced by *M. bovis* strain Mb1

Since macrophage stimulation can be assayed by monitoring the release of cytokines, I tested whether infection of BAMs by *M. bovis* activates production of pro- or anti-inflammatory cytokines. Using Bio-Plex® ELISA, I measured the secretion levels of IL-1, IL-6, IL-10, IL-12, IL-13, IFN-γ, IFN-α, TNF-α, and TGF-β after 3h, 6h, and 24h of incubation of BAMs with *M. bovis*, and the results are shown in figure 2.2. Although untreated and *M. bovis*-treated BAMs had no stimulation of the pro-inflammatory TNF-α cytokine, LPS treatment of BAMs induced significant production at 3h and 6h ($P \leq 0.01$ and $P \leq 0.001$), with the levels dropping at 24h. On the other hand, BAMs infected with Mb1 significantly induced production of IL-10 at 6h. There was a slight drop at 24h compared to un-infected BAMs and LPS-infected BAMs. There was no detectable production of IL-1, IL-6, IL-13, IFN-γ, IFN-α, and IL-12 (data not shown). I also observed large data dispersion in the results. Our data indicate that the infection of BAMs leads to cytokine production skewed towards an anti-inflammatory profile.

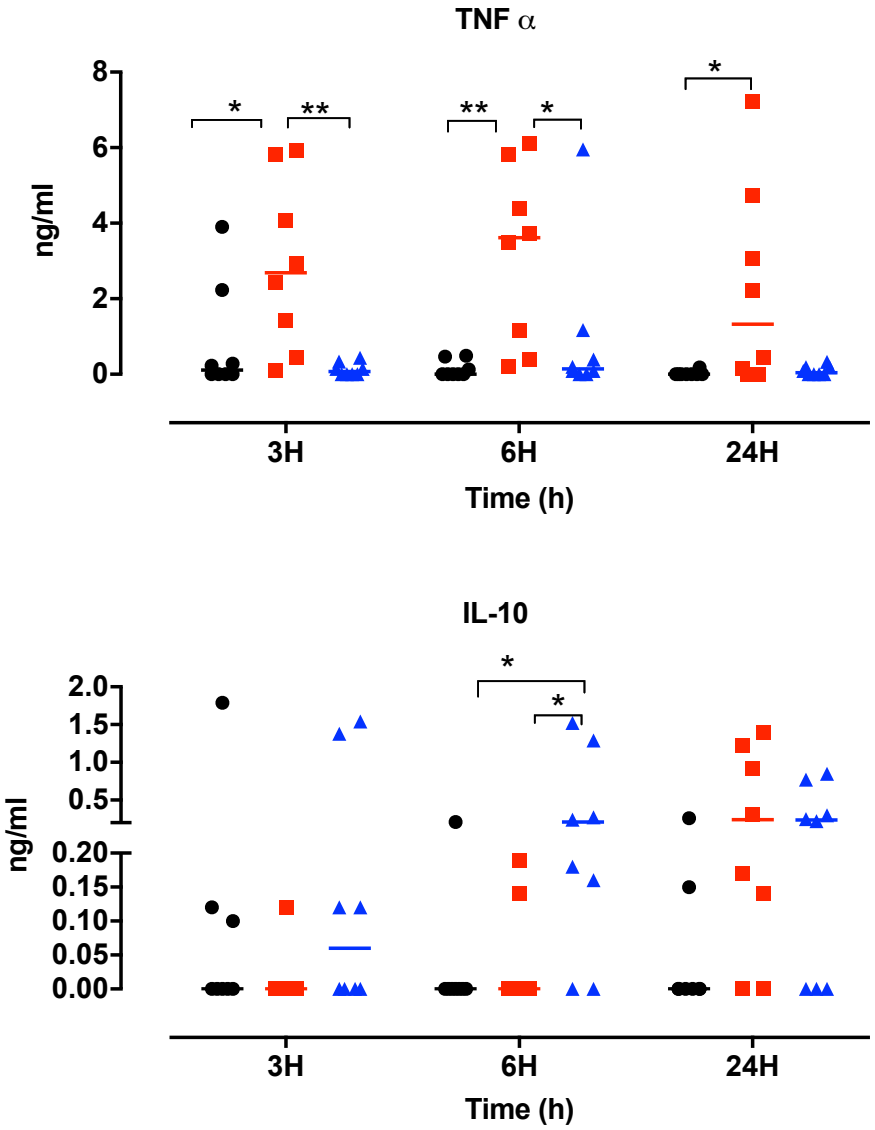


Figure 2. 2: Cytokine ELISA. The quantification of TNF-α and IL-10 in supernatants from bovine alveolar macrophages collected from eight animals after different hours of incubation with *M. bovis* Mb1 is shown. Cytokines were quantified from undiluted supernatants. The left Y-axis is units in ng/ml. Results are presented as median of the values and differences between treatments were determined by one-way ANOVA (Kruskal–Wallis test analysis). Significant differences between the treatments and untreated cells are indicated by * = $P<0.05$, ** = $P<0.01$. Black symbols represent untreated BAMs; blue represents Mb1-infected BAMs; and red represents LPS-treated BAMs.

M. bovis strain Mb1 decreases production of NO in BAMs

Nitric oxide is part of the antimicrobial arsenal for polarized macrophages that leads to inactivation of key enzymes of the target pathogens. NO is produced after metabolism of L-arginine by the iNOS/NOS-2 enzyme to NO and citrulline (MacMicking et al., 1997a). To investigate whether *M. bovis* infection of BAMs modulates their ability to produce NO by iNOS, I examined 24h post-infection the production of NO using the Griess reagent and the results are shown in figure 2.3. Compared to the untreated BAMs, incubation of BAMs with *M. bovis* strain Mb1 resulted in low production of NO although the level of this inhibition was not significant. LPS-treated cells showed significant NO levels at 3h ($P \leq 0.05$). Finally, compared to untreated BAMs, incubation with LPS and infection with Mb1 at 24h resulted in less NO production but this difference was not significant. The results suggest that Mb1 may decrease NO generation by BAMs. Although a control with LPS+Mb1 should have been included.

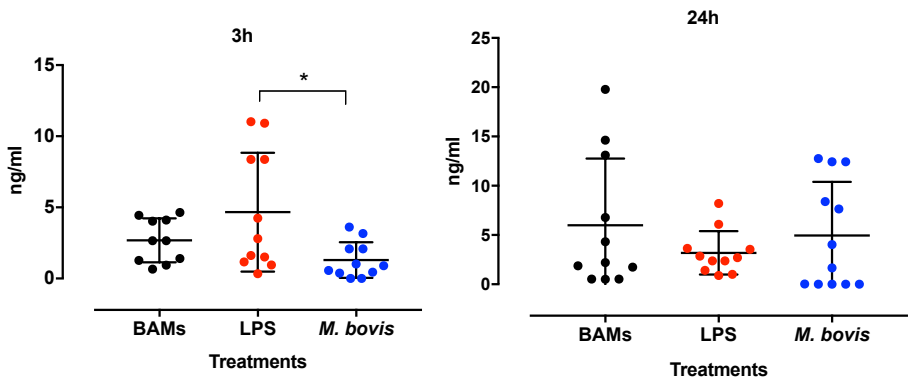


Figure 2. 3: Nitric oxide production. NO quantification was carried out in undiluted supernatants from alveolar macrophages collected from eleven animals after incubation with *M. bovis* for 3 and 24h. The results are presented as median of the NO concentration. LPS was used as a positive control. (LPS: Lipopolysaccharide, *M. bovis*: *M. bovis* strain Mb1). Each dot represents BAMs from an animal. Black symbols represent untreated BAMs; blue represents Mb1-infected BAMs; and red represents LPS-treated BAMs and differences between treatments were determined by one-way ANOVA (Kruskal–Wallis test analysis). Values of $P < 0.05$ were considered significant. Black symbols represent untreated BAMs; blue represents Mb1-infected BAMs; and red represents LPS-treated BAMs.

There was a basal production of NO by the untreated BAMs. This finding is not surprising since NO has an array of functions depending on level and site of production of the cells in the lungs; it can act as a vasodilator, a bronchodilator, a pro-inflammatory and an antimicrobial molecule (Bogdan, 2001).

I further decided to use qPCR to confirm the NO production results observed above (Fig. 2.3). To choose a reference gene for qPCR, the most important factor to consider is the stability of the reference gene. I used the Normfinder™ software to score and rank different reference genes. For cattle, the suggested reference genes were RPS9, RPS15a, YHZ, GAPDH and β-Actin (Kishore et al., 2013). As indicated in the now standardized MIQE guidelines, five untreated and five treated samples were used to run qPCR for all reference gene candidates for each sample (Bustin et al., 2009). The quantification cycle (C_q) was used to calculate a theoretical quantity based on a theoretical standard curve with 100% efficiency with a value of 1 copy at 35 C_q of target RNA in the samples by using the efficiency of each reaction (Equation 1) and the Normfinder™ algorithm was used to calculate sample variation and to produce stability scores. GAPDH and β-Actin were found to have the highest stability score as shown in table 2.1, as they had the lowest stability value in Normfinder™.

I tested for iNOS gene expression changes after each treatment. Also, I tested for expression of TLR-2 and 6 genes, as involved in the invasion of other mycoplasmas in macrophages (Muhlradt and Frisch, 1994; Muhlradt et al., 1997). The results are shown in figure 2.4.

Equation: Theoretical copy number= 10^((Cq-35)/slope of target standard curve)…….(2.1)

Table 2. 2: Estimation of the stability of reference genes using NormFinder™ algorithm. In NormFinder™ a low value indicates a more stable gene.

Gene name	Stability value
GAPDH	0.586
β-Actin	0.810
RPS15a	1.203

RPS9	1.263
YHZ	1.730

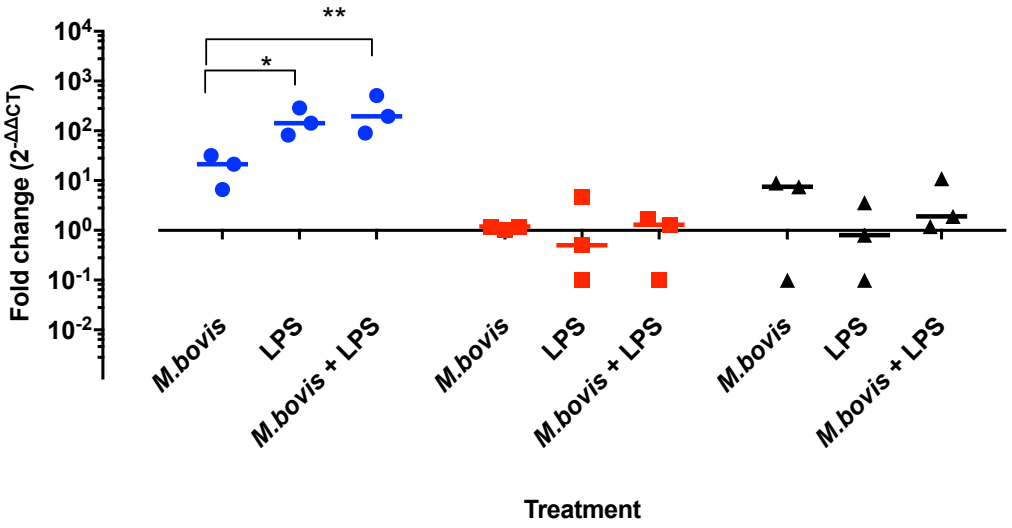


Figure 2. 4: Differential expression of iNOS, TLR-2 and TLR-6 genes in infected BAMs. The expression of these genes was measured by qPCR on untreated BAMs, treated with LPS, *M. bovis*, and LPS + *M. bovis*. Specific primers to iNOS, TLR-2 and 6 were used. The comparative threshold cycle ($2^{-\Delta\Delta CT}$) method was utilized to calculate fold change (mean± SD) between different treatments compared to the untreated control. Data were normalized using GAPDH as the reference gene. Each dot represents a different animal. Black symbols represent TLR-6; blue represents iNOS; and red represents TLR-2. The experiment was conducted with 2 technical replicates. Significant differences between the treatments and Mb1 treated cells are indicated by * = $P<0.05$, ** = $P<0.01$.

There was an increase in the iNOS gene expression after infection with *M. bovis,* LPS and Mb1+LPS compared to untreated cells (Fig. 2.4). Expression of the TLR-2 and TLR-6 genes in BAMs was not changed after the different treatments.

Effect of *M. bovis* strain Mb1 infection on BAMs cell surface receptors associated with antigen presentation

I sought to see if *in vitro* infection with *M. bovis* strain Mb1 would modulate expression of different receptors involved in antigen presentation mechanisms. I used FACS to evaluate the relative expression of various relevant cell surface markers: MHCII; CD163: a scavenger

receptor on macrophages; CD80/86: co-stimulatory signal necessary for T-cell activation; and CD40: a co-stimulatory signal important in APC cells. The results are shown in figure 2.5.

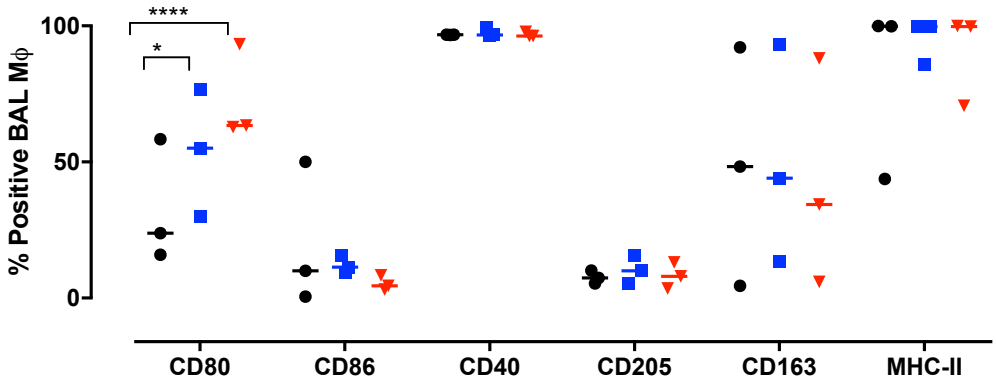


Figure 2. 5: Expression of surface receptors on infected BAMs. Expression of receptors associated with antigen presentation and other macrophage receptors was detected using FACS. Each data point represents a different animal and the experiment was conducted with two technical replicates. Black symbols represent untreated BAMs; blue represent Mb1-infected BAMs, while red represents LPS-treated BAMs. The data are presented as mean and differences between treatments. Significant differences between the treatments and untreated cells are indicated by * = $P<0.05$, *** = $P<0.001$.

The results indicated that there were no statistically significant differences in expression of the APCs surface receptors, CD86, CD40, CD163, CD205, and MHC class II except for the significant overexpression of one of the B7 co-stimulatory molecule, CD80 after Mb1 infection (Fig. 2.5).

2.4. Discussion

During mycoplasma infection, invasion of the respiratory tract results in activation of the innate response as the first line of defense and primes the adaptive immune response against the bacteria. Jungi *et al*., suggested that alveolar macrophage effector functions are modulated upon *Mycoplasma spp.* infections and their inappropriate activation can promote an excessive inflammatory response (Jungi et al., 1996). Several studies have shown that *M. bovis* invades and persists in alveolar macrophages (Burgi et al., 2018; Maina et al., 2018; Suleman et al., 2016a) circumventing their effector functions. The intracellular phase of *M. bovis* represents a protective niche for avoiding clearance by host immune cells as demonstrated by Kleinschmidt *et al*., in alveolar macrophages (Kleinschmidt et al., 2013). To date, the mechanisms of evading macrophage effector functions by *M. bovis* are not well elucidated. I used primary alveolar macrophages infected with Mb1 to demonstrate that the production of NO and cytokines was not stimulated. These experiments aimed to determine whether *M. bovis* modulated host macrophage effector functions and the results from these studies represent pathogen-host interactions that occur between *M. bovis* and cattle alveolar macrophages. Here I report no stimulation of TNF-α and an increase of IL-10 expression in macrophages infected with the *M. bovis* strain Mb1.

Macrophages are an important line of defense against mycoplasmas in the respiratory tract (Caswell, 2014; Maunsell et al., 2011), exerting their protective effects principally through phagocytosis, production of ROS, NO, and inflammatory cytokines (Lai et al., 2010; Lohmann-Matthes et al., 1994; MacMicking et al., 1997b). Disruption of these macrophage functions could lead to persistence and survival of *M. bovis*.

The secretion profile of *M. bovis* in infected primary BAMs indicates basal levels of pro-inflammatory TNF-α and up-regulation of anti-inflammatory IL-10 (Fig. 2.2). Consistent with these findings, there are previous reports of low IFN-γ and TNF-α and high IL-10 cytokines expression in monocytes (Mulongo et al., 2014). TNF-α plays a key role in inflammatory, infectious, and malignant conditions while IL-10 plays a role in suppression of inflammation and skews the immune response to a TH_2 response dominated by antibodies (Couper et al., 2008a). These observations agree with reports of a TH_2-skewed immune response after an *M. bovis* infection resulting in moderate IFN-γ, high IL-4 levels in T-cells; and high IgG1, low IgG2 titres in the serum (Vanden Bush and Rosenbusch, 2003). In contrast, Rodriguez and his group used

immunohistochemistry to identify increased *in vivo* levels of TNF-α, IL-4, and IFN-γ in experimentally infected lungs compared to un-infected controls. In *M. bovis*-infected neutrophils, there was stimulation of the pro-inflammatory cytokines TNF-α and IL-12 (Jimbo et al., 2017). Interestingly, just as I observed by ELISA tests, there was no significant production of IL-1α, IL-1β, IL-2, IL-6, and IL-8 between the different treatments (Rodriguez *et al*., 2015). In our previous work, we show evidence that suggests that there was *in vitro* expression of IL-17 after incubation of PBMCs with *M. bovis* Mb1 proteins (Prysliak et al., 2018) however I found that there was no IL-17 production by infected primary BAMs. The different observations may be attributed to the specific cell types used in different studies.

The pro-inflammatory cytokines IFN-γ and TNF-α are known to be stimulated by expression of iNOS (MacMicking et al., 1997a). The low NO levels secreted by infected primary BAMs could be explained by the low expression of TNF-α in these cells. Only a few papers have been published regarding inhibition of NO production by mycoplasma. Here I report a trend reduction of NO production (Fig. 2.3) and no reduced expression of the iNOS gene (Fig. 2.4) by infected primary macrophages at 3h compared to untreated cells. Similarly, there is evidence of host immune modulation of bovine neutrophils by *M. bovis*. *In vitro* studies revealed an increase in elastase release and inhibition NO production after infection of neutrophils with the *M. bovis* strain Mb1 (Jimbo et al., 2017). Additionally, *M. bovis* inhibited neutrophil respiratory burst (Thomas et al., 1991). As described below inhibition of NO production is not only observed in *M. bovis*.

During infection of rat macrophages, *Francisella tularensis* uses phase variation to alter the host macrophage nitric oxide response and its antigenicity. A reverse phase shift reduces NO and restores intracellular growth perhaps to prolong the host-pathogen interaction during acute or to establish a chronic state. Given that *M. bovis* also has the phase-variation phenomenon I cannot rule out the likelihood of this survival strategy (Cowley et al., 1996). Similarly, infection of the macrophage cell lines, J774-A1 and RAW 264.7 with the parasite *Toxoplasma gondii* resulted in inhibition of NO production through up-regulation of arginase 1 (Arg1) which competes with iNOS for the substrate L-arginine. This effect on the cell lines was concluded to be a survival mechanism to avoid clearance (Cabral et al., 2018). There are very few studies on arginase 1 activity during mycoplasma infection. A study carried out in sheep with contagious agalactia

demonstrated significant decreases in host erythrocyte arginase activity and significant increases in plasma NO compared to healthy sheep. The study further suggested that increase in plasma NO might contribute to the elimination of the pathogen (Hanedan et al., 2017). Other pathogens deploy their own arginases for immune suppression, such as, *Helicobacter pylori* arginase that inhibits nitric oxide production in the cell line RAW 264.7 (Gobert et al., 2001) and a parasite-encoded arginase of *Leishmania spp*. that increases disease outcome of murine cutaneous leishmaniasis through decreased iNOS (Gaur et al., 2007). In another study in the J774 macrophage-like cell line infection with BCG (Bacille Calmette-Guerin) suppressed NO production by induction of Arg1 (El Kasmi et al., 2008). Currently, there is no known information on the effects of bovine host macrophage Arg1 modulation with infection of mycoplasma or if there is a gene that encodes for Arg1 in any mycoplasma.

In contrast, *in vivo* co-localization of *M. bovis* antigens and iNOS-producing macrophages was observed by IHC of lungs from infected calves compared to un-infected calves. The findings suggested that NO was also somewhat involved in the lung damage seen in severe chronic *M. bovis* infection. The discrepancies observed in the different *M. bovis* studies may be due to presence of other types of macrophages such as tissue macrophages and the fact that host-protective functions of iNOS/NO are restricted to certain stages of the diseases. As such, in infections with mycobacteria, iNOS is critical in the late and not early stages of the infection (Cooper et al., 2000).

In other mycoplasmas, *M. synoviae* (Lavric et al., 2007), *M. fermentans* (Muhlradt and Frisch, 1994) and *M. hyopneumoniae* (Bai et al., 2013) are known to induce NO production in macrophages leading to increased acute disease progression in their hosts. These differences with *M. bovis* may be a result of strain-specific differences and also due to the stage of infection.

Additionally, high levels of NO production are found in healthy lung tissues of different species (sheep, human and rats), (Radi et al., 2001). Here, I also reported basal production of NO in untreated BAMs (Fig. 2.3).

One could also argue that the presence of TLRs 2 and 6 (Fig. 2.4) genes transcripts on primary BAMs helps in recognition of PAMPs and leads to the transcription of genes involved in the activation of the innate host defense, mainly the production of pro-inflammatory cytokines such

as TNF-α, IL1β, and IL-12 and NO. Several studies on *M. genitalium*, *M. fermentans*, and *M. pneumoniae* indicate that following stimulation of TLR-2 and TLR-6, the NF-κB signalling pathway is activated to elicit inflammatory responses (Into et al., 2004b; McGowin et al., 2009; Shimizu et al., 2005, 2008). Expression of surface receptors on APCs is important in antigen presentation to the adaptive immune system. In this study, when comparing the surface markers expression on the primary BAMs populations (Fig. 2.5) there were no significant changes in the expression of MHC II, CD163, CD86, CD40 markers on infected primary BAMs except for CD80 that was significantly up-regulated in infected primary BAMs. These results did not give us information on what modulates primary BAMs surface-receptors expression during antigen presentation to T-cells. Thus, in chapter 3, I investigate if there is *in vivo* modulation of presentation of the T-cell antigen OVA to T-cells after infection with *M. bovis* strain Mb1.

2.5. Conclusion

This chapter describes how *M. bovis* has successfully evolved different strategies to modulate BAMs effector functions for survival and persistence. As a result of this, I report modulation of pro-inflammatory cytokine production with a decrease trend in NO production and an increased expression of one of the B7 complex receptors, CD80 in *M. bovis* infected primary BAMs. This is one of the different strategies applied by *M. bovis* for survival and immune suppression of the host. The ability of the immune cells to move within the host also allows pathogens to disseminate systemically inside their host to various tissues such as, synovial membrane (Haines et al., 2001b), liver (Adegboye et al., 1995), and kidneys (Hermeyer et al., 2012b). Taken together, the findings provide evidence for the suppression of macrophage effector functions and have implications in disease pathogenesis.

Transition statement

Modulation of protein activity by post-translational modification through cellular phosphorylation is an important regulatory mechanism that can be evaluated by using a high-throughput fashion in a kinome arrays. Kinome arrays are a useful tool to identify biochemical pathways modulated by host-pathogen interactions. Elucidation of signal transduction pathways by kinome array is becoming popular because kinase-mediated phosphorylation is the predominant mechanism for regulation of protein function. (Manning et al., 2002). Bovine-specific peptide kinome arrays is available (Jalal et al., 2009), and it was used in search of Toll-like receptor signalling in bovine monocytes (Arsenault et al., 2009), and to unravel novel immune evasion mechanisms of Mycobacterium avium subsp. paratuberculosis in monocytes (Arsenault et al., 2012; 2013). In a related study, our research team investigated phosphorylation pathways modulated by the interaction of bovine alveolar macrophages and monocytes cells with M. bovis Mb1 using a kinome array (Mulongo et al., 2014). Among the pathways that were increased phosphorylated were: IFN-γ, apoptosis, and NF-κB, while the down-phosphorylated pathways were: caspases, T-cell receptor signalling, inhibition of cellular proliferation, and antigen processing. In this study, we sought to analyse in more detail the antigen presentation capacity of infected macrophage cells.

CHAPTER 3. Antigen presentation capacity of primary BAMs infected with *Mycoplasma bovis* strain Mb1

3.1 Abstract

A successful antigen presentation requires multiple molecular signals. Antigen-presenting cells (APCs) capture and process antigens into small peptides before displaying them on their surface in association with MHC class I and II molecules and thus allowing the naïve T-cells to recognize them and induce an immunological reaction. Other co-stimulatory molecules expressed on the APCs must bind to their respective receptors found on T-cells. CD40 receptors on APCs bind to the CD40L found on the T-cells. Only then, the host mounts an immune response to that antigen characterized by the production of IL-2 that induces T-cell proliferation followed by a cascade of events. I showed that the expression of several key BAM surface markers *in vitro*, (chapter 2) was not affected by infection with Mb1 except for CD80 and hence, I sought to determine if the ability of BAMs to present a known T-cell antigen (ovalbumin) was affected after infection with *M. bovis.* An animal trial was designed to test the humoral and cell-mediated immune responses to ovalbumin (OVA) followed by a challenge with *M. bovis* strain Mb1. The animals were vaccinated twice with OVA formulated in Emulsigen^{TM} and CpG oligodeoxynucleotides. At the end of the trial, I pulsed the BAMs with OVA co-incubated with T-cells and collected supernatants and cells for cytokine and proliferation assays respectively to test the hypothesis that infection of the BAMs with *M. bovis* Mb1 would reduce/interfere with the presentation of OVA to PBMCs and T-cells. The study resulted in a marked humoral response in the two groups (infected and control), as judged by the steady increase of anti-ovalbumin antigen titers over the course of vaccination. Examination of haematoxylin and eosin (H&E)-stained lung tissues sections indicated that there were no differences in morphology and cell infiltration of the bronchiolar lumen with neutrophils or macrophage accumulation of cells between the two groups. There was also no effect on the T-cell proliferative responses to the antigen of either the infected nor the non-infected BAMs except for the cells stimulated with ConA.

3.2 Materials and Methods

Bacteria strains and culture conditions

The experiments were conducted using *M. bovis* strain Mb1, which was previously isolated from

the synovial fluid of a calf exhibiting signs of arthritis (Perez-Casal and Prysliak, 2007). Cultures were grown in modified Hayflick's medium at 37°C in a 5% CO_2 atmosphere. Bacteria were collected by centrifugation at 10 000 × *g* and suspended in the same medium at the highest concentration as determined by counting the bacteria in a hemocytometer. Challenge doses were prepared by serial dilutions of the original suspension in the same medium to 5.00×10^{10} cfu/ml.

Animal trial

All animal experiments followed the guidelines of the Canadian Council on Animal Care and followed protocols approved by the University of Saskatchewan Animal Research Ethics Board. The trials were conducted at the VIDO-InterVac research farm. The trial consisted of 6 beef calves about 6 months old previously determined to be negative for respiratory mycoplasmas by nasal swab cultures. Six calves were assigned into 2 groups (infected and non-infected) of 3 animals each using a random numbers table. The groups were separated for the duration of the study. All six animals were vaccinated with 1mg of OVA in 30% Emulsigen™, 250µg/dose of CpG, and PBS intramuscularly as described in figure 3.1. On day 57 *M. bovis* strain Mb1 (5×10^{10} cfu/ml) suspended in 4ml of culture medium was administered directly into the trachea of animals in one group of the calves (infected group). The control group was given an equal volume of sterile Hayflick's broth. Animals were observed daily for clinical signs of disease and euthanized on day 60. Investigators who were unaware of the status of the animals carried out all clinical and gross pathology assessments. Necropsy of all animals and collection of tissue samples was carried out as described before (Prysliak et al., 2011) on day 60. Serum antibody titres against the OVA antigen were determined at days 0, 21 (prior to boost), 41, (second boost), 57 (pre-challenge), and 60 (post-challenge).

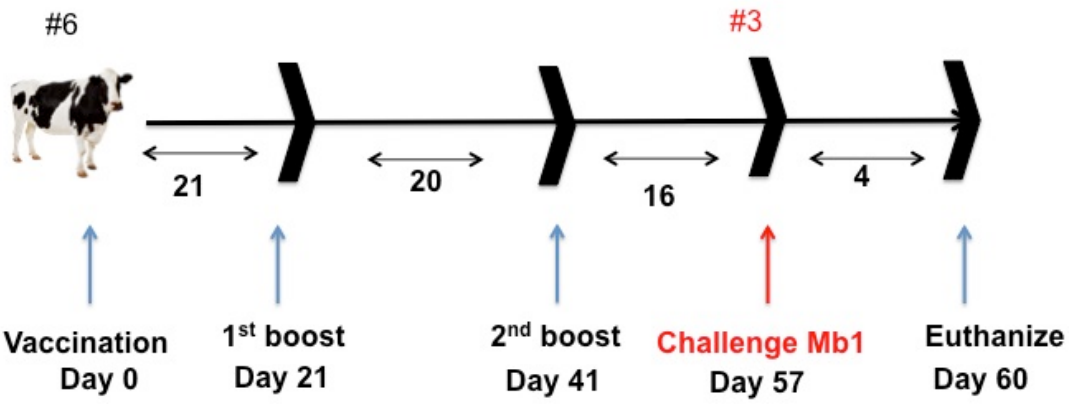


Figure 3. 1: Animal trial scheme.

Sample collection and preparation:

Recovery of *M. bovis* from tissue samples

Nasal secretions were collected on day 0 and after the trial (D60) and lung sections after euthanizing the animals, after a 48h pre-enrichment in Hayflick's medium, they were spread on plates for isolation of *M. bovis*. Colonies resembling *M. bovis* were isolated and their genomic DNA extracted by detergent lysis and proteinase K treatment. Using polymerase chain reaction (PCR) primer were specific of the *M. bovis* gene encoding glyceraldehyde 3-phosphate dehydrogenase, GAPDH was used to identify *M. bovis* strain Mb1 (Perez-Casal and Prysliak, 2007). The primer pair used is shown in table 3.1. PCR reactions of 25 µl final volume were carried out using a commercial Taq-polymerase and buffer (GE-Healthcare Bio-Sciences Inc., Baie d'Urfe, QC) with 3mM $MgCl_2$. PCR conditions used were 1 cycle of 2min at 94°C, 35 cycles of 30s, 94°C, 30s annealing at the appropriate temperature for the primer combination used, 2min extension at 72°C and a final cycle of 5min at 72°C An aliquot of the sample was processed and bands resolved by gel electrophoresis on a 2% agarose gel stained with ethidium bromide and a positive control of *M. bovis* DNA was included on the gel.

Genomic DNA isolation

Briefly, *M. bovis* cells were harvested using trypsin/ ethylene diamine tetra acetic acid (EDTA) buffer. Cells were washed twice in phosphate-buffered saline and lysed with 0.5xTBE (25 mM TRIS, 25 mM boric acid and 0.5 mM EDTA) containing 0.25% (v/v) NP-40). RNase H (0.5 mg/ml, Sigma Inc, St. Louis, MO, USA) was added and samples incubated for 45min at

37°C followed by addition of Proteinase K (0.5 mg/ml, Sigma Inc, St. Louis, MO, USA) with continued incubation for another 45min at 37°C. The cell debris was removed by centrifugation at 14 000 rpm at 4°C for 10min. The supernatants were transferred into new Eppendorf™ tubes and DNA extracted using 500 µl of buffered phenol (Sigma Inc, St Louis, MO, USA) and with chloroform/isoamyl alcohol (24:1, Sigma Inc, St. Louis, MO, USA). Addition of 5 M NaCl to a final concentration of 200 mM and 2 volumes of ice-cold 100% ethanol precipitated the DNA. The samples were mixed by inverting them and further precipitated at −80°C for 20min or −20°C overnight. The DNA was pelleted by centrifugation at 14 000 rpm at RT for 20min, washed with ice cold 70% ethanol and pelleted again and allowed to air-dry. The pellet was resuspended in 40µl of TE, pH 7.5 and used for the PCR reaction.

Table 3. 1: Primers used in this study.

Name	Sequence	Product size (bp)	Reference
M.b.gap7	F: ATAGGAGGATCCAAAAGAGTCGCTATCAATGG TTTTGGACG	1018	(Perez-Casal and Prysliak, 2007)
M.b. gap8	R: GGAAATGGTACCTTACTTAGTTAGTTTAGCAAA GTATGTTAATG		(Perez-Casal and Prysliak, 2007)

The sequences of GAPDH primers used in the trial are shown. F: Forward primer and R: Reverse primer.

Serum

Blood was collected from all animals by jugular venipuncture on days 0, 21 (prior to boost), 41, (second boost), 57 (pre-challenge), and 60 (post-challenge). Serum was decanted following centrifugation at 1000 x g for 15min to remove clots, red blood cells and other cells. Serum was then aliquoted and stored at -20°C until analysis for OVA IgG antibody titres.

Isolation of bovine alveolar macrophages (BAMs)

The lungs of the recently euthanized animals were gradually filled via the trachea with sterile Hanks balanced salt solution (HBSS) supplemented with 2X antibiotic-antimycotic cocktail (penicillin, streptomycin, and amphotericin B) and 1X gentamicin. The lungs were gently massaged and the lavage decanted into sterile bottles to harvest the BAMs suspension. Washing of the lungs required 3 to 4l of HBSS (dependent upon lung size) and about one-half of this volume could be recovered. The macrophage suspension was centrifuged at 400 × *g* for 10min at 4°C and the cell pellet was collected and washed with cold PBS supplemented with 2X antibiotic-antimycotic and suspended in D-MEM medium. The cells were then counted in a Coulter counter and the suspension diluted to the required working concentration of BAMs. D-MEM containing 10% FBS, 0.05 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 0.1mM nonessential amino acids, and 10mM HEPES buffer was used to culture BAMs for all subsequent assays. Trypan blue 0.4% and flow cytometry were used to assess the viability of the BAMs.

Lung tissues samples for histological and immunohistological analysis

Lung tissues of approximately $3cm^3$ were excised and immersed in 10% buffered formalin (10% formaldehyde in PBS; v/v) for transport to PDS (Prairie Diagnostics Lab, SK, Canada) for wax embedment. The formalin-fixed tissues were hand-trimmed and wax-embedded into labelled cassettes. Three-micron sections were then cut from the wax-embedded tissues using a microtome (Leica Microsystems, Milton Keynes, UK) and floated on the surface of a pre-heated (45-55^oC) water bath. The floating sections were picked up using a microscope slide (VWR, West Sussex, UK) ready for IHC.

PBMC purification

Blood samples (60 ml from each animal) were collected in syringes containing sodium EDTA and the cells separated by centrifugation at 2500rpm for 20min at RT, with no brake. The buffy coat fraction (containing PBMC) was purified by Ficoll gradients (GE Healthcare, ON, Canada). The PBMCs isolated were washed thrice with PBSA (137 mM NaCl, 2.7 mM KCl, 7 mM Na_3PO_4, and 1.5 mM KH_2PO_4) containing EDTA and suspended in MEM medium in the working concentration.

T-cell isolation

Isolated PMBCs were labelled with a cocktail of biotin-conjugated antibodies for 5min in the refrigerator (2-8°C). The cocktail contained antibodies against CD8, CD14, CD15, CD16, CD19, CD36, CD56, CD123, TCR γ/δ, and CD235a (Glycophorin A) (Miltenyi Biotec, Auburn, CA). $CD4^+$ T-cell MicroBead cocktail was added to the cell suspension and further incubated for 10min in the refrigerator (2-8°C). Next, the $CD4^+$ T-cells were negatively selected for on LS columns using a magnetic isolated cell sorter (Miltenyi Biotec, Auburn, CA). The collected flow-through containing unlabeled cells represented the enriched $CD4^+$ T-cells. The cells were washed twice in PBSA and suspended in MEM medium in the correct concentration.

Proliferation assay

The isolated PBMC and T-cells were used for the proliferation assay with stimulation of the cells with concanavalin A (ConA; Sigma-Aldrich, Ontario, Canada), different OVA concentrations and/or *M. bovis* Mb1. 96-well tissue culture plates were seeded with PBMC or T-cells (5 x 10^5/per well) and incubated at 37°C in 5% CO_2 atmosphere in the presence of 1μg of ConA/ml and/or infection with *M. bovis* Mb1 using 2.5 x 10^6 cfu/ml at a MOI of 5:1 at 24h in triplicates. A solution containing 0.4 μCi of [^{3}H] thymidine (GE Healthcare)/well was added, and the cells were incubated for 18h. The cells were harvested (Filtermate Harvester; Guelph, Ontario), the amount of incorporated [^{3}H] thymidine was determined in a scintillation counter (Top Count NXT; Guelph, Ontario), and the stimulation index was determined by the formula: counts per minute of treated PBMCs/counts per minute of control or unstimulated PBMCs

CFSE assay

The enriched T-cell population was stained with CellTrace CFSE staining solution (Cell Trace CFSE, ThermoFisher, Massachusetts, USA), and incubated for 20min in a 37°C water bath. CFSE is a cell-permeant fluorescent intracellular labeling tool of live cells and monitors lymphocyte proliferation. Cells were collected by centrifugation and suspended in MEM medium and plated in a 96 well U-bottom plate for incubation with: ConA, live *M. bovis* Mb1 with, or without ConA, 0.5μg/ml, 1μg/ml, 5μg/ml, and 10μg/ml of OVA) and assessed proliferation with flow cytometry. Samples were analysed with (BD FACSCalibur™, San Jose, CA) and data analysed with Kaluza® (Beckman Coulter, Indianapolis, US).

Humoral and cell mediated immune responses assay

Serum antibodies responses (IgG) to OVA antigen were determined using a multiplex platform as previously described (Prysliak et al., 2018). Briefly, 96-well Immuno II plates were coated with 5ng OVA per well in carbonated buffer overnight at 4°C. The plates were washed 4 times in PBS-T to prevent non-specific binding to the plastic surface and the wells were coated with PBST-G for 2h. After washing, serum samples from the 6 animals were serially diluted in PBST-G (1/100 first and 4-fold further on) placed in the wells and incubated for 2h at RT and washed thrice. IgG was detected using alkaline phosphatase-conjugated goat anti-bovine IgG (KPL antibodies, MA, USA) followed by washes. Alkaline-phosphatase substrate NTA was added and the reaction incubated for 30min at RT and the A_{405} was read using a Bio-Rad Xmax spectrophotometric microplate reader. The titers were calculated by the intersection of least-square regression of A_{405} versus the logarithm of the serum dilution.

Purification of bovine PBMC and proliferation assay were also performed as described above on days 0 (Pre-vaccination), pre-boost, pre-challenge and post-challenge. T-cells were further purified from the PBMCs using MACS® cell separation kit (Miltenyi Biotec, CA, USA), as described above on day 60 (post-challenge) according to the manufacturers' instructions. The purified T-cells were used in the *in vitro* antigen-presentation assay.

***In vitro* antigen presenting assay**

The BAMs isolated from the 6 animals were pulsed with different concentrations of OVA for 4h and 8h then an equal number of T-cells from the same animals were added. The supernatants were collected after 24h, 48h, and 72h of incubation at 37°C in 5% CO_2 atmosphere and used for determination of cytokine levels. The T-cell proliferation assay was used as readout for successful antigen presentation using a thymidine incorporation assay alongside a CFSE assay by flow cytometry. Samples were analysed with (BD FACSCalibur™, San Jose, CA) and data analysed with Kaluza®

Haematoxylin and eosin (H&E) staining of tissues

The Prairie Diagnostic Services Inc. (SK, CA) performed the H&E staining, and I collected the slides for subsequent microscopic examination and photography.

Immunohistochemistry (IHC) of tissue sections

Sections were immersed in 100% xylene (2 x 6min each) then immediately transferred to 100% ethanol (2 x 6min each) and finally placed in a 100^{o} C water bath for 15min. Antigen retrieval was achieved by immersing the sections in pre-heated citrate buffer [0.25% (w/v) trisodium citrate hydrate ($Na_3C_6H_5O_7$) and 0.025% citric acid ($C_6H_8O_7$); pH 6.1] in deionized water. The sections were heated at 800 watts in a microwave for 10min then left to cool in citrate buffer for a further 10min before transferring them into water for 2min. IHC was carried out on the samples using the VECTASTAIN®ABC-AP (alkaline phosphatase) detection kit according to the instructions of the manufacturer and stained at 1/500 dilution using *M. bovis* antibodies (procedure performed by PDS), mouse anti bovine TNF-α (in-house), mouse anti bovine MHC II (Serotec), and mouse anti bovine IFN-γ (in house). An isotype control (mouse IgG1) was used as a negative control. The sections were dehydrated by sequential immersion in 70%, 90%, and 100% ethanol respectively and subsequent immersion in 100% xylene. Sections were then mounted with coverslips using VectaMount™ (Vector, CA, USA) and left overnight to dry and subsequently examined under a microscope and photographed.

Statistical analysis

Prism 7.0c (GraphPad Software, Mac OS X, La Jolla, CA, USA) was used for data analysis. I used one-way ANOVA (Friedman) to analyse the serum OVA IgG and Ag presentation assay data and two-way ANOVA to analyse the PBMC and T-cell proliferation data. This was followed by multiple-groups analysis using Dunn's multiple comparison tests for the serum OVA IgG and Tukey's multiple comparison tests for the proliferation data to compare the different treatments.

3.3. Results

Isolation of *M. bovis* Mb1

Nasal swabs from all the 6 animals were collected in saline and cultured in PPLO medium to detect *M. bovis* Mb1. I prepared genomic DNA from the cultures and used primers specific for *M. bovis* (Table 3.1). I was not able to isolate *M. bovis* Mb1 from the nasal swab cultures as judged by the negative results of the PCR reactions performed on samples collected before and after the *M. bovis* Mb1 challenge (data not shown).

Lung pathology

All the animals were vaccinated with OVA but only one group was infected with Mb1. Macroscopic examination of the lungs from the non-infected and infected group were performed and the results are shown in figure 3.2. No gross pathological characteristic of *M. bovis* infection such as the characteristic multifocal 'marble cheese' caseous necrosis effect and consolidation of the lungs was observed at necropsy of lungs of the infected group of calves; #186, #187, and #190. The pathological scores were determined by detecting the presence of lesions, multifocal white nodules containing caseous material, characteristic of *M. bovis* infections or consolidation of the lung tissues. There was only 1% pathology score on one of the lung tissue (261) in the non-infected (control group).

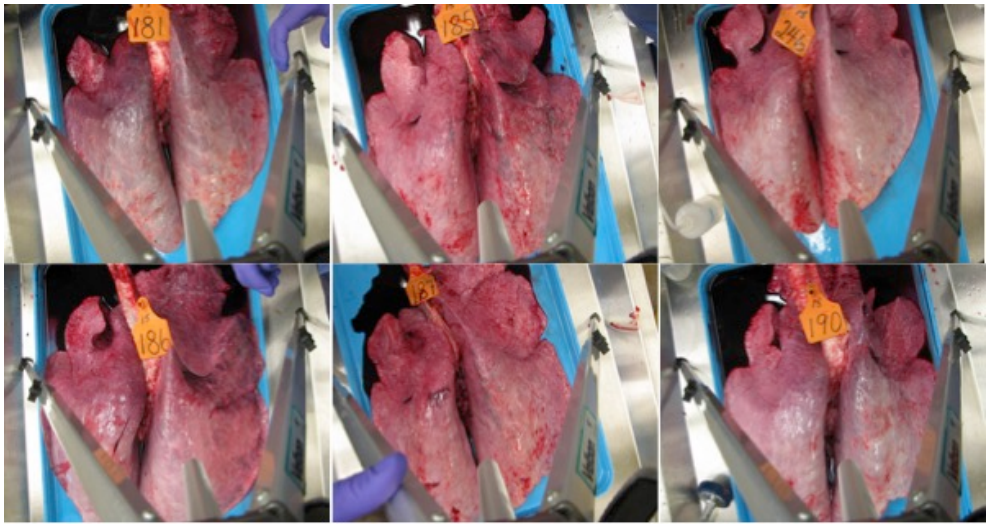


Figure 3. 2: Figure 3.2: Lung pathology. Gross pathology of lungs from all the six animals vaccinated with OVA, 3 infected with *M. bovis* Mb1: #186, #187, and #190 and 3, control non-infected calves: #181, #185 and #246 in the trial.

Haematoxylin and eosin (H&E) staining of the infected lung tissues

H&E staining was also carried out for all the 6 animals and the results are shown in figure 3.3. There were no differences in morphology and cell infiltration of bronchiolar lumen with neutrophils and macrophages by either group. The results infer that the lungs in both groups (infected and un-infected) had the same basal cell infiltration.

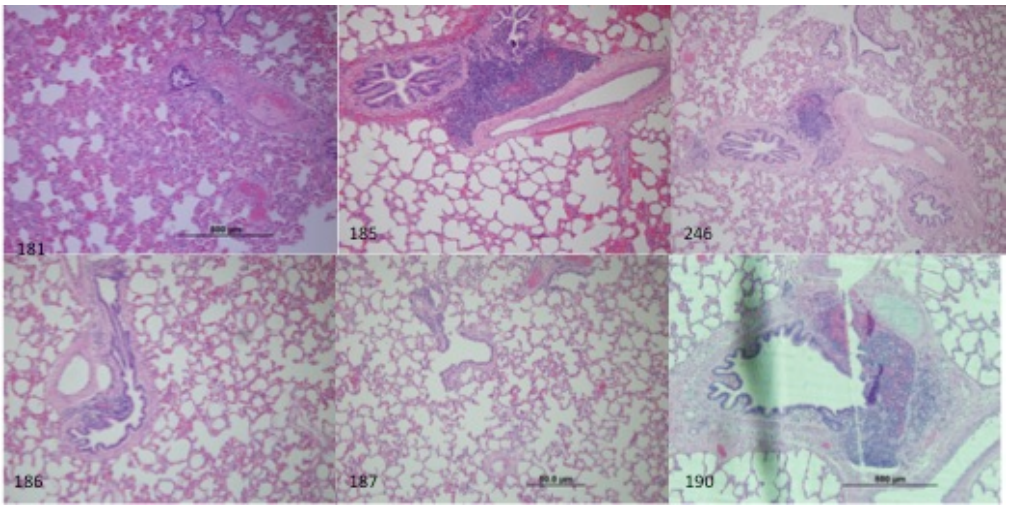


Figure 3. 3: H&E staining of tissue sections of infected and non-infected lungs. The H&E staining was from both non-infected: #181, #185, and #246 and infected cattle: #186, #187, and #190.

Immunohistochemistry (IHC) of infected lung tissue sections

IHC was performed on lung sections and the results are shown in figure 3.4. TNF-α and MHC II staining were positive for all the 6 animals and appeared as a brown deposit in BAMs that adhered to capillary endothelium (Figs. 3.4a and b). This brown deposit was absent in the isotype control stained sections. There were no differences in the intensity of the staining between the infected and non-infected groups. Staining for *M. bovis* and IFN-γ was negative in all the lungs (Fig. 3.4c).

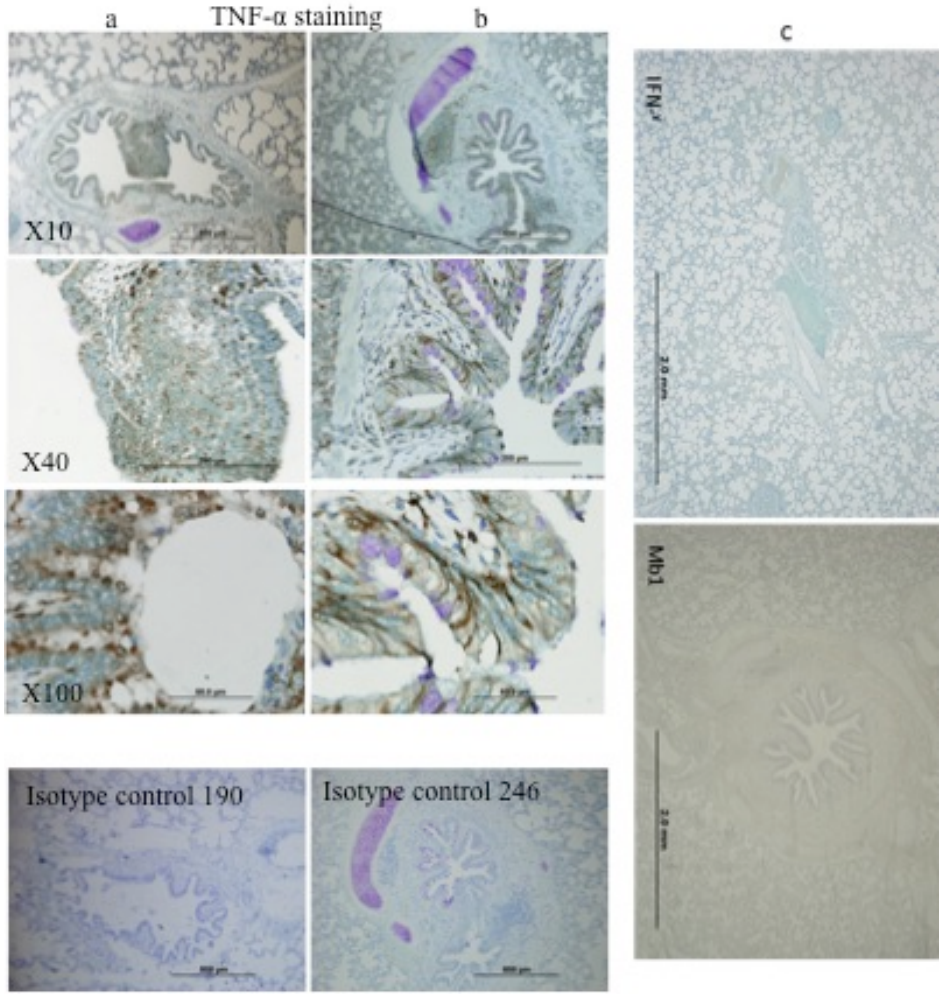


Figure 3. 4: IHC staining of paraffin embedded lung tissues from representative lungs. Paraffin sections of lungs from infected and non-infected cattle were stained for TNF-α, IFN-γ, MHC II, and *M. bovis*. As shown in panel (a) and (b), there were cells that stained positive (brown deposit) for TNF-α for both infected and non-infected animals (190 and 246 respectively). In contrast to (c), the panel shows no positive stain for either Mb1 and IFN-γ.

Humoral immune responses to OVA antigen

I measured serum total IgG to OVA antigen and the results are shown in figure 3.5. As expected, there was a steady increase of titers over the course of vaccination, with the serum titers in both groups (infected and un-infected) higher after the 2nd vaccination boost and before the Mb1 challenge compared to day 0 values. However, despite not been significant there was a slight increase in titres of the infected group after the challenge compared to the un-infected group.

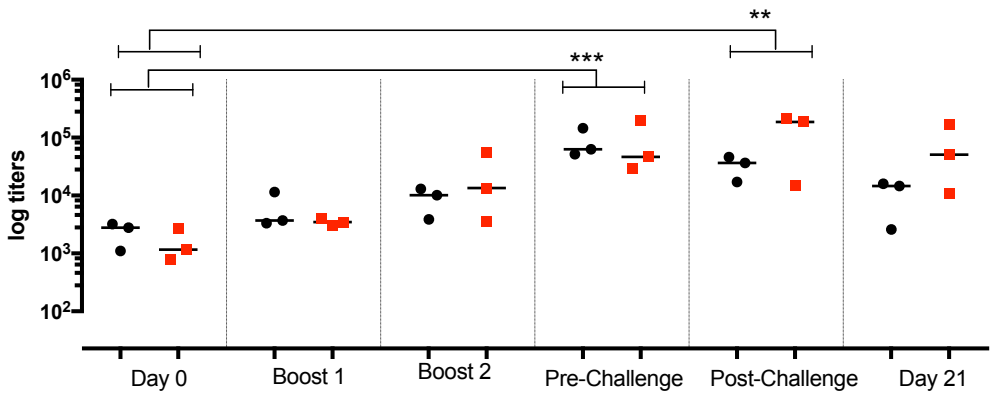


Figure 3. 5: Serum immune responses to OVA antigen. The mean of the IgG titers against OVA is shown as a bar across the symbols. Blood samples were collected pre-vaccination (Day 0), after vaccination (Day 21), after the 2 boosts and prior to *M. bovis* challenge (Pre-challenge) and at the time of necropsy (post-challenge). The differences between treatments were determined by one-way ANOVA (Friedman test, Dunn's multiple comparison tests). Significant differences between the treatments and day zero are indicated by ** = $P<0.01$, *** = $P<0.001$.

Cellular-mediated immune responses to the recall antigens

PBMC proliferation was monitored in response to recall antigens OVA and Mb1 over the course of the vaccination and infection and the results are shown in figure 3.6. Compared to day 0 (blue bar), stimulation indexes were higher after the first boost (Day 21, black bar) in both panels, (a) control un-infected and (b) infected samples (Fig. 3.6) pre- and post-vaccination samples. This trend was not seen after the second boost or post-challenge except in recall stimulation with 10µg/ml OVA post-challenge (purple bar) in control un-infected samples where there was an increase in stimulation index even though not significant (Fig. 3.6a).

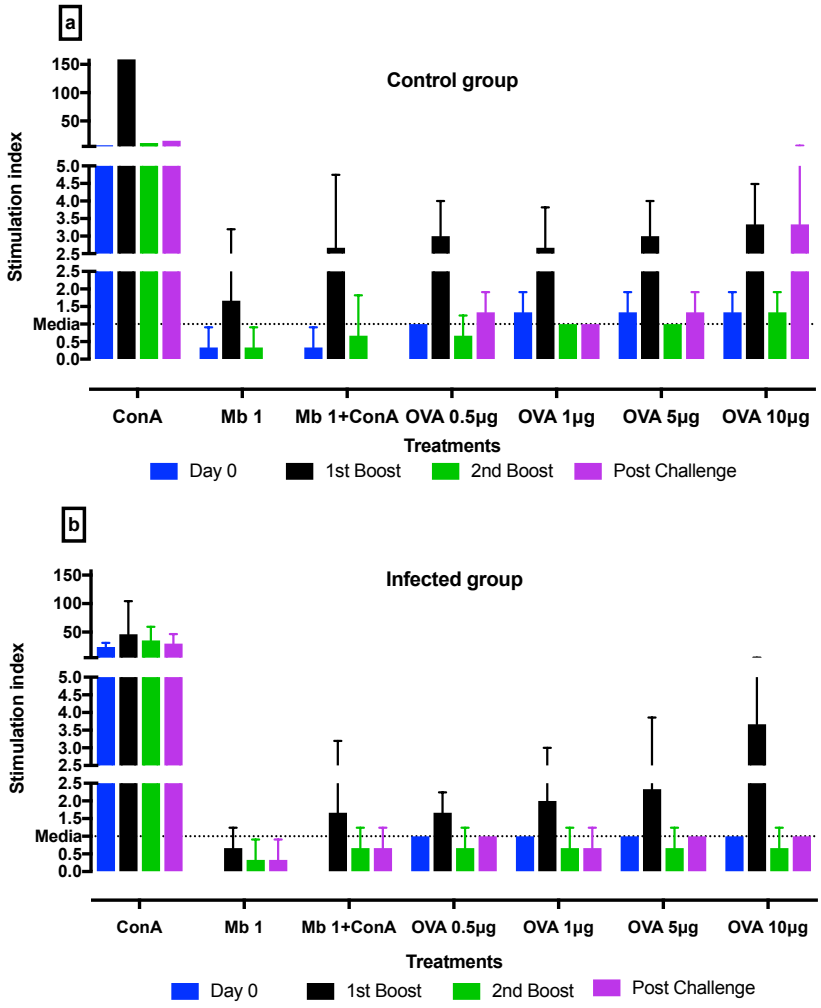


Figure 3. 6: PBMC proliferation assay in the course of the animal trial. 2 groups; control (a) and infected (b), 3 animals in each group. Compared to day 0 incubation of live, *M. bovis* Mb1 with or without ConA, 0.5µg/ml, 1µg/ml, 5µg/ml, and 10µg/ml of OVA with bovine PBMC from 6 cattle there was an increase after the 1st boost but not after the second and post-challenge except stimulation with 10µg/ml of OVA in the control un-infected group. The differences between treatments were determined by two-way ANOVA (Tukey's multiple comparisons test). Blue: day 0, black: 1st boost, green: 2nd boost and purple: post challenge.

Previous results have shown that the ability of Mb1 to suppress the host immune system could be accomplished by the down-regulation of lymphocyte proliferation and hence could explain the lack of proliferation seen in the infected animals even exposure to live Mb1 (Mulongo et al., 2014). Post-challenge proliferation of purified T-cells was also performed by flow cytometry

using the CFSE staining to confirm the results observed in the thymidine incorporation assay and the results are shown in figure 3.7. As shown in proliferation of total purified T-cell post-challenge (Fig. 3.7), there was no significant T-cell proliferation observed regardless of the recall OVA antigen concentration used to stimulate in either the control or challenged group compared to the untreated cells. There was significant proliferation in ConA and ConA+Mb1 T-cells compared to untreated cells and all treatments compared to ConA but no differences between the un-infected and infected samples in the trial.

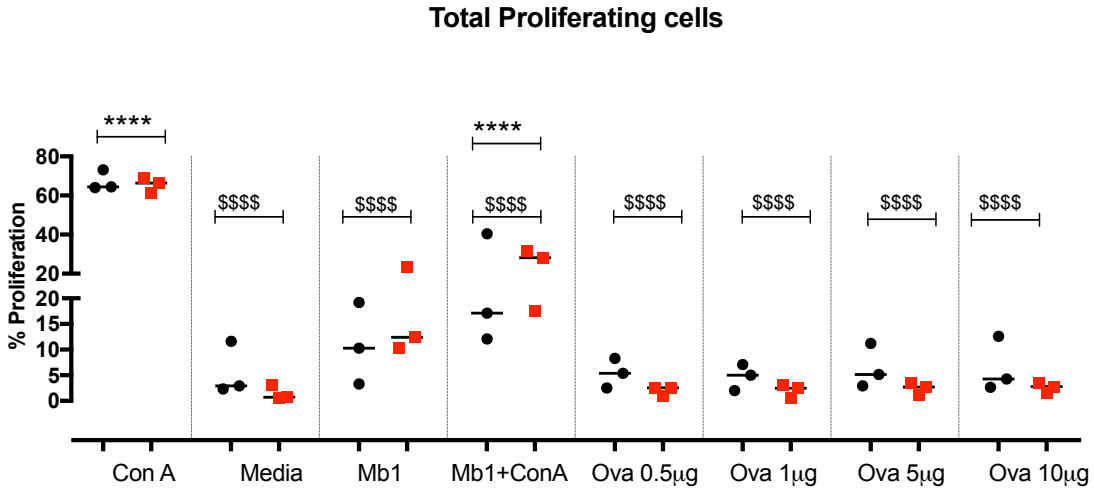


Figure 3. 7: Post-challenge purified T-cell Proliferation (CFSE). This is a graph representative of the post-challenge purified T-cells proliferation. Compared to untreated cells, incubation of live, *M. bovis* Mb1 with or without ConA, 0.5µg/ml, 1µg/ml, 5µg/ml, and 10µg/ml of OVA with purified T-cells from the 6 calves there was no significant stimulation of proliferation. Significant differences between the treatments and untreated cells (medium) are indicated by * = $P<0.05$, *** = $P<0.001$ and between treatments and ConA are indicated by $$$$= $P<0.0001$. Black represents non-infected animals and red represents infected animals.

M. bovis Mb1 modulates BAMs presentation of ovalbumin antigen to T-cells

Macrophages and T-cells were co-cultured to investigate the stimulatory capacity of Mb1 infected macrophages, to promote T lymphocyte proliferation. The results are shown in figure 3.8. In the *in vitro* OVA antigen presentation assay where purified T-cells were co-incubated with OVA pulsed macrophages for 4h and 8h there was no T-cell proliferation by either OVA antigens and Mb1 compared to untreated cells. The results suggest an inhibitory proliferation mechanism

by Mb1compared to Mb1+OVA. There were significant stimulation indices by ConA compared to untreated cells showing that the cells were not dead during the assay (Fig. 3.8).

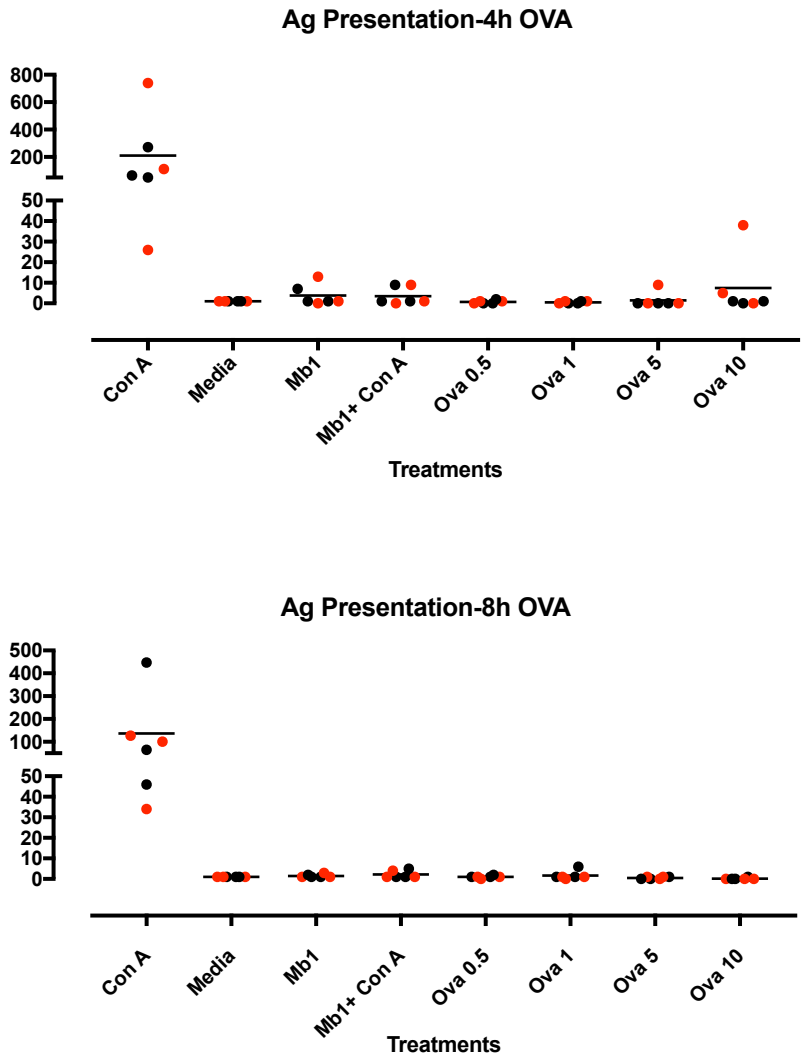


Figure 3. 8: OVA antigen presentation assay. BAMs incubated with different concentrations of OVA for 4h and 8h and mixed with an equal number of purified T-cells. Cells were incubated for 72h the cells and pulsed with thymidine for 18h. No stimulation was observed regardless of the stimulation except for ConA. Significant differences between the treatments and (untreated cells) medium are indicated by *** = $P<0.001$. Red represents infected animals and black represents non-infected animals.

3.4. Discussion

Antigen presentation is a biological process where the APCs capture and process antigens into small peptides before displaying them on their surface in association with MHC class I and II molecules, and hence allow the T-cells to recognize them and induce an immunological reaction (Ten Broeke et al., 2013).

MHC I molecules are expressed on all nucleated cells and present the endogenous proteins (self or from intracellular pathogens) to $CD8^+$/cytotoxic T-cells. They are made of α and β_2-microglobulin chains of proteins. The antigens are processed by proteasomes into peptides (8-10 residues) and transported across the ER by transporter proteins (Yaneva et al., 2010). The peptide fragments that fit the MHC I groove binds and the complex is expressed on the cell surface to be presented to cytotoxic T-cells (Yaneva et al., 2010). If the $CD8^+$ T-cells recognize self they do not mount an immune response but if they recognize foreign peptides they will kill the infected cells (Li and Raghavan, 2010).

MHC II molecules are expressed on activated macrophages, dendritic cells and activated B-cells. They are made up of α and β chains of protein produced in the cytoplasm and injected into the endoplasmic reticulum (ER) where they are loaded with an invariant chain protein in their groove to avoid binding to proteins in the ER. The invariant chain also guides the MHC II molecules to the endosomes within the Golgi apparatus where they are loaded with the antigen peptide and presented on the APCs surfaces (Yaneva et al., 2010). The antigen processing by APCs occurs when the antigen is enclosed in a phagosome that later merges with the endosome and specific endosomal and lysosomal enzymes present in the endosome (e.g. cathepsins), dice the exogenous proteins into small peptides (10-30 residues) and subsequently destroy the invariant chain which leads to peptide loading on to the MHC II groove. Eventually the MHC II plus the antigen complex are transported to the cell surface for display to $CD4^+$ cells (Ten Broeke et al., 2013).

Three steps are required for successful antigen presentation after processing of the antigen: first, the peptides bound to MHC molecules are presented to the TCR complex; second, the CD80/86 complex on the APCs binds to CD28; and third, CD40 binds to the CD40 ligand (CD40-L) on the T-cells. Only then, the host mount an immune response to the antigen that is characteristic of the

production of IL-2 and T-cell proliferation followed by a cascade of events (Mantegazza et al., 2013).

T-cells express on their surface the T-cell receptor (TCR) that allows presentation of peptides, foreign or self bound to MHC by APCs such as macrophages. The recognition of processed antigen through MHC molecules to TCR is the most important step towards successful antigen presentation (Sundberg et al., 2007). TLR stimulation in macrophages has also been demonstrated to be important during microbial presentation on MHC II molecules (Iwasaki and Medzhitov, 2004).

To determine whether antigen presentation of OVA was modulated by an *M. bovis* infection, an animal trial was designed to test the humoral and cell-mediated immune responses to OVA before and after a challenge with *M. bovis* strain Mb1 (Fig. 3.1). The experiments used freshly isolated primary BAMs, PBMCs, and T-cells from infected and non-infected calves that were vaccinated with OVA. The responses after stimulation of OVA-antigen-specific, or ConA-driven T-cells were compared. In chapter 2, I demonstrated that CD80 receptors were upregulated after infection with Mb1, while there were no differences in expression of MHC class II, CD163, CD86 or CD40 receptors on both infected and non-infected primary BAMs (Fig. 2.5) There was increased T-cell proliferation after incubation with ConA showing that the cells used were viable. On the other hand, there was no proliferation after incubating with the other recall antigens, OVA and Mb1 except for at the highest OVA concentration (10µg/ml), on the post-challenge samples in the three control un-infected samples (Fig. 3.6a).

Several previous studies have shown that proliferation of lymphocytes is down-regulated after infection by *M. bovis* but not their ability to produce cytokines both *in vitro* (Suleman et al., 2016a; Thomas et al., 1990; van der Merwe et al., 2010; Vanden Bush and Rosenbusch, 2002) and *in vivo* (Mulongo et al., 2013; Prysliak et al., 2013). Similarly, there are previous reports on the decreased proliferative capacity of lymphocytes in response to incubation with phytohemagglutinin, a mitogen, by various mycoplasma strains and species *in vitro* (Naot et al., 1977). It remains to be discerned whether this is due to faulty antigen presentation, lack of co-stimulation or T-cell exhaustion due to persistent antigen stimulation.

We also know that T-cell activation and proliferation is influenced by control of the PD-1/PD-L1 immune checkpoint. The programmed death 1 (PD-1) receptors are expressed on activated T-cells and their interaction with the PD ligand 1 (PD-L1) which is expressed on activated macrophages (Butte et al., 2007). Interaction of the PD-1/PD-L1 complex leads to T-cell signalling exhaustion inducing immunosuppression (Wherry, 2011). We previously demonstrated that there was intermediate and high expression of PD-1 in $CD4^+$ and $CD8^+$ on *M. bovis* infected cells compared to un-infected cells and that blocking the interaction of PD-1 with its ligand with an anti-PD-1 antibody restored normal T-cell proliferation (Suleman et al., 2018). These results indicate that *M. bovis* infection is involved in the PD-1/PD-L1 inhibitory pathway modulation and immune exhaustion causing impaired host immune responses.

The animal trial resulted in significant increases of the IgG responses to OVA. Compared with day 0 titres, there were significant humoral responses to the OVA antigen in serum samples obtained from all animals (infected and non-infected) before and after challenge (Fig. 3.5). The current evidence also suggests that there was a failure to infect the three calves as judged by the negative result of the PCR reactions for *M. bovis* DNA post-challenge.

To successfully test the Ag-presenting model there is need to reproduce the infection through challenge models. Previous, *M. bovis* intranasal challenge (5×10^8 cfu/ml) disease models established in our lab included a co-infection model with BHV-1 to reproduce the disease (Prysliak et al., 2011). I used the *M. bovis* strain Mb1 for an intratracheal challenge (5×10^{10} cfu/ml) for this experiment to infect the calves. In my study, I did not report any lung gross pathology (Fig. 3.2) in the Mb1-infected claves nor did I detect *M. bovis* antigens in the lungs of any calves by IHC (Fig. 3.4c). A number of studies have previously reported a reproducible infection using a *M. bovis* alone challenge model with *M. bovis* doses ranging from 10^9 to 10^{10} cfu/ml and 2-3 weeks infection period after the challenge (Dudek et al., 2016; Nicholas et al., 2002; Zhang et al., 2014). Since the aim of the study was to investigate the modulation of BAMs' ability to present antigens after infection by *M. bovis* strain Mb1, I did not use the co-challenge model of infection as studies have shown that previous exposure to viruses such as BHV-1 and BVDV were immunosuppressive to the host (Prysliak et al., 2011). I also used the adjuvant formulation with Emulsigen™ and CpG2007 used previously in *M. bovis* vaccine trials (Mulongo et al., 2013; Prysliak et al., 2013).

3.5. Conclusion

Taken all together, these results suggest that although vaccination with OVA antigen was successful, the challenge using *M. bovis* Mb1 alone did not result in disease. The failure of the challenge could be explained by the small window between infection before euthanising (4 days) not being enough to observe infection and pathology. The antigen presentation capacity of the primary BAMs was also not successfully detected as there were no differences between the control un-infected and infected Mb1 samples.

Transition statement

We have previously shown that Mb1 delays apoptosis in primary bovine PBMC subsets (van der Merwe et al., 2010), monocytes (Mulongo et al., 2014) and in primary bovine macrophages (Suleman et al., 2016a). Mulongo et al., has further shown that in bovine monocytes, Mb1 decreases caspase 9 expressions and activates translocation of NF-κβ-p65 to the nucleus (Mulongo et al., 2014). In this present study, I sought to elucidate the macrophage apoptosis pathways that are modulated by M. bovis Mb1; for this, I chose to use BoMac, a bovine macrophage cell line (Stabel and Stabel, 1995). My decision to switch from using primary alveolar macrophages for the apoptosis assays to a cell line was based on the variability of the results obtained from BAMs isolated from abattoirs in the cytokine and NO assays. Commercial cattle are not inbred, and thus there are several factors including genetics, age, or previous exposure to bacteria or viruses that may influence the results. The advantage of the BoMac cell line is that it is genetically uniform and that a large number of cells can be readily grown. BoMac cells were a generous gift from Dr. Matthias Schweizer (Universität Bern, Switzerland). Before using the BoMac cells, I needed to confirm the results observed with primary alveolar macrophage cells that Mb1 delays STS-induced apoptosis. I tested for the survival of Mb1 in the cell line, cytokine profiles after infection and whether Mb1 affects STS-induced apoptosis.

CHAPTER 4. Modulation of apoptosis and activation of the NF-kappa beta-signalling pathway by *Mycoplasma bovis* Mb1

Citation

Maina, T., Prysliak, T., Perez-Casal, J., 2019. *Mycoplasma bovis* delay in apoptosis of macrophages is accompanied by increased expression of anti-apoptotic genes, reduced cytochrome C translocation and inhibition of DNA fragmentation. Veterinary Immunology and Immunopathology 208, 16-24.

Copyright

My contributions

This paper is 90% part of this chapter's data. I did all the work (collection of data, analysis and manuscript writing), Prysliak and Perez-Casal helped in editing the manuscript.

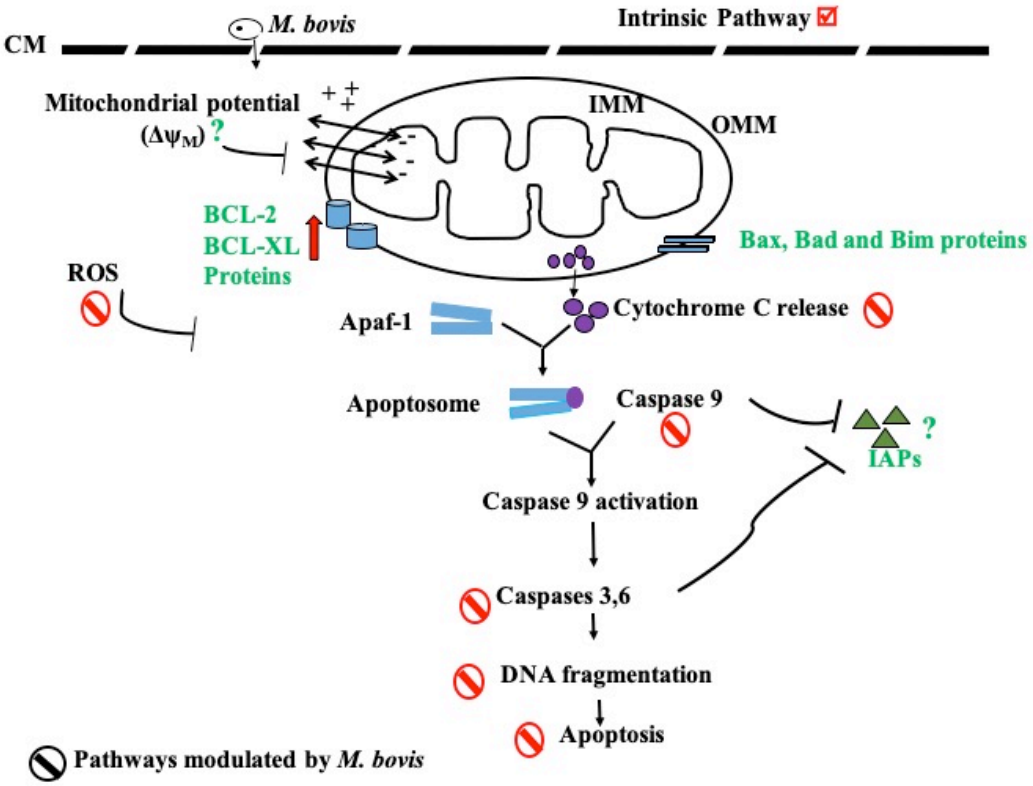
4.1. Abstract

Mycoplasma bovis is a pathogen that causes various diseases including chronic bronchopneumonia, mastitis, otitis, keratoconjuctivitis, meningitis, infertility, and arthritis in cattle (Arcangioli et al., 2008; Caswell and Archambault, 2007; Caswell et al., 2010). The importance of *M. bovis* among other pathological agents implicated in bovine respiratory disease (BRD) complex has also been shown (Maunsell and Donovan, 2009; Maunsell et al., 2011). BRD is a multifactorial disease of feedlot cattle that is caused by *M. bovis* co-infection with bovine herpes virus (BHV-1); parainfluenza virus (PI-3); bovine respiratory syncytial virus (BRSV); bovine viral diarrhea virus (BVD), and *Mannheimia haemolytica*, and *Pasteurella multocida* (Arcangioli et al., 2008). It is also the most pathogenic bovine mycoplasma in Europe and North America (Nicholas and Ayling, 2003; Snowder et al., 2006). Although *M. bovis* is considered to be an extracellular pathogen, there is *in vivo* (Kleinschmidt et al., 2013; Prysliak et al., 2011) and *in vitro* evidence that indicates intracellular presence and survival in different subsets of the host immune cells (Burki et al., 2015; Maeda et al., 2003; van der Merwe et al., 2010). To date, the mechanisms by which *M. bovis* interacts with host cells to generate disease are poorly

understood. Naturally occurring *M. bovis* persists in a herd over an extended period and can be consistently identified not only in lesions but also commonly in healthy and pneumonic lungs (Gagea et al., 2006b).

Apoptosis (Programmed cell death) is employed by host cells to limit pathogen survival and propagation. Apoptosis can also be exploited by the pathogen to evade the host immune response (Fulda et al., 2010; Hotchkiss et al., 2009; Renehan et al., 2001). *M. bovis* has been demonstrated to exert both pro and anti-apoptotic effects on a diversity of cell types, including neutrophils, lymphocytes, monocytes and macrophages (Jimbo et al., 2017; Mulongo et al., 2014; Suleman et al., 2016a; van der Merwe et al., 2010). In monocytes, *M. bovis* manipulates apoptotic signalling through inhibition of caspase 9 and activation of the NF-kβ pathway (Mulongo et al., 2014). *M. bovis* strain Mb1 exerts anti-apoptotic effects on macrophage STS-induced apoptosis (Suleman et al., 2016a) but the mechanisms are unknown. In this study, I used the BoMac bovine macrophage cell line to determine the apoptosis pathways modulated by Mb1. The BoMac cell line has been previously used to study *M. bovis* co-infection with bovine viral diarrhoea virus in bovine macrophages (Burgi et al., 2018). In the present study, I demonstrate the ability of *M. bovis* strain Mb1 but not Mb304 to delay STS-induced apoptosis in BoMac. I also show that *M. bovis* strain Mb1 activates the NF-kβ pathway and induces up-regulation of pro-survival genes Bcl-2 and Bcl-X_L; and inhibits the activity of caspases 3, 6, and 9, reduces ROS production and DNA fragmentation upon infection. Our data continue to support observations that *M. bovis* inhibit apoptosis for survival to potentially facilitate bacterial survival, replication and transmission.

Graphical Abstract



4.2. Materials and Methods

Bacteria strains and culture conditions

The experiments were conducted using *M. bovis* strain Mb1, which was previously isolated from the synovial fluid of a calf exhibiting signs of arthritis (Perez-Casal and Prysliak, 2007), and the *M. bovis* strain Mb304 obtained from bison lung tissue (Suleman et al., 2016a). Cultures were grown in modified Hayflick's medium at 37°C in a 5% CO_2 atmosphere. Bacterial cells were collected by centrifugation (5,500 x g for 15 min) at the exponential phase of growth and then washed with minimum essential medium (MEM; Invitrogen, Burlington, ON, Canada). Bacteria were suspended to a cell density of $1x10^8$ cfu/ml in MEM supplemented with 30% glycerol and stored at -70°C until use.

BoMac cell line

The bovine peritoneal macrophage cell line (BoMac) was obtained as a gift from Dr. Matthias Schweizer (Universität Bern, Switzerland) and cultured in D-MEM supplemented with 1mM sodium pyruvate and 10% FBS. The cell line was grown to near confluence and split every third-day using trypsin/EDTA. The cell viability was evaluated by the trypan-blue exclusion assay as described earlier (van der Merwe et al., 2010).

Mycoplasma bovis invasion and survival in BoMac cells

The assay was carried out as previously described with a few modifications (Burgi et al., 2018; Hegde et al., 2014; Suleman et al., 2016a; van der Merwe et al., 2010). Briefly, 5 x 10^6/per well BoMac cells were infected with a range of MOIs (BoMac: *M. bovis*) of 1:0.1 and 1:5, using 5 x 10^6 cfu/ml and 2.5 x 10^7 cfu/ml respectively. After 3h incubation, the cells were washed and cultured in medium containing 400µg/ml Gm (Gibco®, Sigma Inc, St Louis, MO, USA) for 2h to kill any extracellular bacteria. Cells were washed twice with warm D-MEM, suspended in D-MEM and incubated for 2, 6, and 18h treatment as shown in the scheme (Fig. 4.1a). Cells were cultured in the presence or absence of Gm to ensure that the use of Gm did not interfere with the recovery of viable intracellular bacteria. The culture supernatant was collected at each time point and the adherent cells harvested and washed with PBS before lysis. The culture supernatant and lysed BoMac cells from each time point were plated in duplicates as 10-fold serial dilutions on

Hayflick's agar plates and incubated for 4 days at 37°C in a 5% CO_2 atmosphere, to determine the number of extracellular and intracellular viable bacteria respectively.

Apoptosis assay

Apoptosis was detected using the Alexa Fluor®488 annexin V/Dead Cell Apoptosis kit (Molecular Probes, Life Technologies®, USA). Briefly, cells (5 x 10^6/per well) were infected with both strains of *M. bovis* (alive and dead) using 2.5 x 10^7 cfu/ml of *M. bovis* at a MOI of 1:5 (BoMac: *M. bovis*) for 24h and incubated in D-MEM medium in the presence or absence of STS (2µg/ml for 6h). To assess if live cells were needed to modulate apoptosis, Mb1 and Mb304 were killed with Gm (400µg/ml) for 2h and washed once with warm D-MEM medium prior to the apoptosis assay. STS (2µg/ml for 6h) was used as positive control, and the negative control included untreated cells grown in the same culture conditions. The cells were harvested using trypsin/EDTA buffer and suspended at 2x10^6 cells/ml in annexin V binding buffer. For every 100µl aliquot of cells, 5µl of annexin V- Alexa Fluor®488 and 1µl of 100µg/ml propidium iodide (PI) were added and incubated for 15min in the dark. Finally, 400µl of annexin V binding buffer was added to each tube, mixed gently and the cells immediately analysed by flow cytometry (BD FACSCalibur™, San Jose, CA) using Kaluza® software (Beckman Coulter, Indianapolis, US).

Caspase assay

BoMac cells were independently infected with both strains at a MOI of 1:5 (BoMac: *M. bovis*) for 24h in the presence or absence of STS. Controls included cells treated with STS (2 µg/ml for 6h) or with V-ZAD-FMK, an apoptosis inhibitor (40µM, for 24h), after which the cells were lysed for 10min in chilled lysis buffer. The cell debris was removed by centrifugation and the cytosolic fraction transferred into a new tube. The protein concentration was adjusted to 200µg/ml with the cell lysis buffer provided in the colorimetric kit (Abcam, Cambridge, United Kingdom). Aliquots of 50µl of the cleared lysate were placed into a 96-well round-bottom microtiter plate, Costar™ (Thermo Fisher Scientific, Massachusetts, US) before addition of 50µl of the 2x reaction buffer and 5µl of the substrates for caspases 3, 6, or 9. After incubation at 37°C for 2h in the dark, the samples were read in a microplate reader (xMark microplate spectrophotometer; Bio-Rad, Philadelphia, USA) at 405nm with a 490nm reference filter.

ROS measurement

Intracellular reactive oxygen species (ROS) was measured using the ROS-ID™ Total ROS Detection kit (Enzo Life Sciences, Inc., Plymouth Meeting, PA) following the manufacturer's instructions. BoMac cells (5 x 10^6/per well) were infected with both strains of *M. bovis* using 2.5 x 10^7 cfu/ml at a MOI of 1:5 (BoMac: *M. bovis*) in the presence and absence of 50µM pyocyanin, a ROS inducer. Pyocyanin-treated cells were used as a positive control and untreated cells as a negative control. After 30min of incubation at 37°C with 5% CO_2 atmosphere intracellular ROS production was immediately assessed by measuring intracellular green fluorescence at excitation 490nm/emission520nm using flow cytometry (BD FACSCalibur™, San Jose, CA) and data analyzed with Kaluza® software (Beckman Coulter, Indianapolis, US).

Cytochrome *C* assay

The cytochrome *C* releasing assay was carried out following the manufacturer's instructions (Abcam, Cambridge, United Kingdom). Briefly, BoMac cells (5 x 10^6/per well) were either un-infected or infected with both strains of *M. bovis* using 2.5 x 10^7 cfu/ml at a MOI of 1:5 (BoMac cells: *M. bovis*) for 24h in the presence or absence of STS (6h) and harvested in ice-cold PBS. Cells were collected by centrifugation and the pelleted cells were suspended in buffer A on ice for 10min. The cells were homogenized with 40 passes by using a Kontes douncer tissue grinder. The homogenates were centrifuged and the supernatants saved as the cytosol fractions. The pellets were suspended in buffer B, vortexed for 10s and kept as the mitochondrial fractions. These two fractions were separated on 12% polyacrylamide gel and subjected to western blotting using a bovine cytochrome *C* antibody (Abcam, Cambridge, United Kingdom) and an IRDye® 800CW goat anti-mouse IgG (LI-COR®, Lincoln, US).

DNA fragmentation

The DNA fragmentation assay was adopted from (Dumont et al., 1999). Cells were infected with both strains of *M. bovis* for 3h in the presence or absence of STS (2µg/ml). Cells treated with STS alone (2 µg/ml, 6h) were used as control. Cells were harvested using trypsin/EDTA buffer. Cells were washed twice in phosphate-buffered saline and lysed with 0.5xTBE (25 mM TRIS, 25 mM boric acid and 0.5 mM EDTA) containing 0.25% (v/v) NP-40). RNAse H (0.5 mg/ml) was added and samples incubated for 45min at 37°C followed by addition of

proteinase K (0.5mg/ml, Sigma Inc, St. Louis, MO, USA) with continued incubation for another 45min at 37°C. The cell debris was removed by centrifugation at 14 000 rpm at 4°C for 10min. The supernatant was transferred into a new Eppendorf tube and, the DNA fragments were separated on a 1.5% (w/v) agarose gel (50 V for 2h), stained with ethidium bromide in TBE buffer and visualized under ultraviolet light.

NF-kβ p65 nuclear translocation assay

This assay was carried out as previously described (Mulongo et al., 2014). Briefly, both strains of *M. bovis* were used to infect 5 x 10^6/per well BoMac cells using 2.5 x 10^7 cfu/ml at a MOI of 1:5 (BoMac: *M. bovis*) and incubated for 24h. The controls included 1µg/well of *Escherichia coli* LPS (Sigma Inc, St. Louis, MO, USA) for 24h, 2µg/well of STS for 6h and untreated cells for 24h. The NF-kβ activation kit (Five Photon Biochemicals, San Diego, CA) was used to separate the cytoplasmic and nuclear fractions. The fractions were separated on a 12% SDS-PAGE and transferred to a nitrocellulose membrane. Western blots were performed using the bovine anti-P65 and Alexa 680 anti-rabbit antibodies provided in the kit. The only modification was that no Tween-20 was added in the blocking step as the image was taken using the LI-COR ®Odyssey® Imager.

Quantitative real-time PCR

Total RNA was extracted from BoMac cells using Trizol® (Life Technologies, Inc., Plymouth Meeting, PA) from five independent experiments. Treatments included cells infected with both strains of *M. bovis*, treated with STS, and un-infected cells as control. RNA quantity was determined using a Nanodrop spectrophotometer and integrity was assessed using denaturing agarose gel electrophoresis. Reverse transcription was performed using primers in Table 4.1; they all had a primer efficiency of above 90%. qPCR was carried out using the RT² SYBR Green Fluor (Qiagen, CA, USA) fast method performed on an iCycler i5 system. The reactions were carried out in duplicate using 5ng of cDNA. The melting curve analysis suggested a single amplicon product. The data are expressed as fold changes as calculated using the $2^{-\Delta\Delta CT}$ method relative to the geometric mean of two stable housekeeping genes, GAPDH and β-actin.

Table 4. 1: Primers used in this study.

Gene	Sequences (5`- 3`)	Amplicon size (bp)	Origin
BAX	F: GCTGCAGAGGATGATCGCAGCTGTG R: ATCAACTCGGGCACCTTGGTGCA	174	(Buhler et al., 2016)
Bcl-2	F: ACGGAGGCTGGGACGCCTTT R: AGGGTGATGCAAGCGCCCAC	121	(Buhler et al., 2016)
Bcl-x_L	F: CACTGTGCGTGGAAAGCGTA R: AAAGTGTCCCAGCCGCC	127	(Valdez et al., 2005)
GAPDH	F: TTCAACGGCACAGTCAAGG R: ACATACTCAGCACCAGCATCAC	119	This thesis
β-actin.	F: AGGCATCCTGACCCTCAAGTA R: GCTCGTTGTAGAAGGTGTGGT	95	This thesis

The sequences of apoptotic gene primers used in the study. F: Forward primer and R: Reverse primer.

Statistical Analysis

Prism 7.0c (GraphPad Software, La Jolla, CA, USA) was used for data analysis. The analyses of caspase activity, relative percentages of cell death and differential gene expression were carried out by using one-way analysis of variance (ANOVA) for multiple-groups analysis. Differences were considered significant it the *P* value was 0.05 or lower.

4.3. Results

M. bovis invades and survives in BoMac cells

To investigate whether our *M. bovis* strains Mb1 and Mb304 can invade BoMac cells, a similar approach of viable intracellular counts analyzed using the GM resistance assay was used (Burgi et al., 2018; Suleman et al., 2016a). The results are shown in figure 4.1.

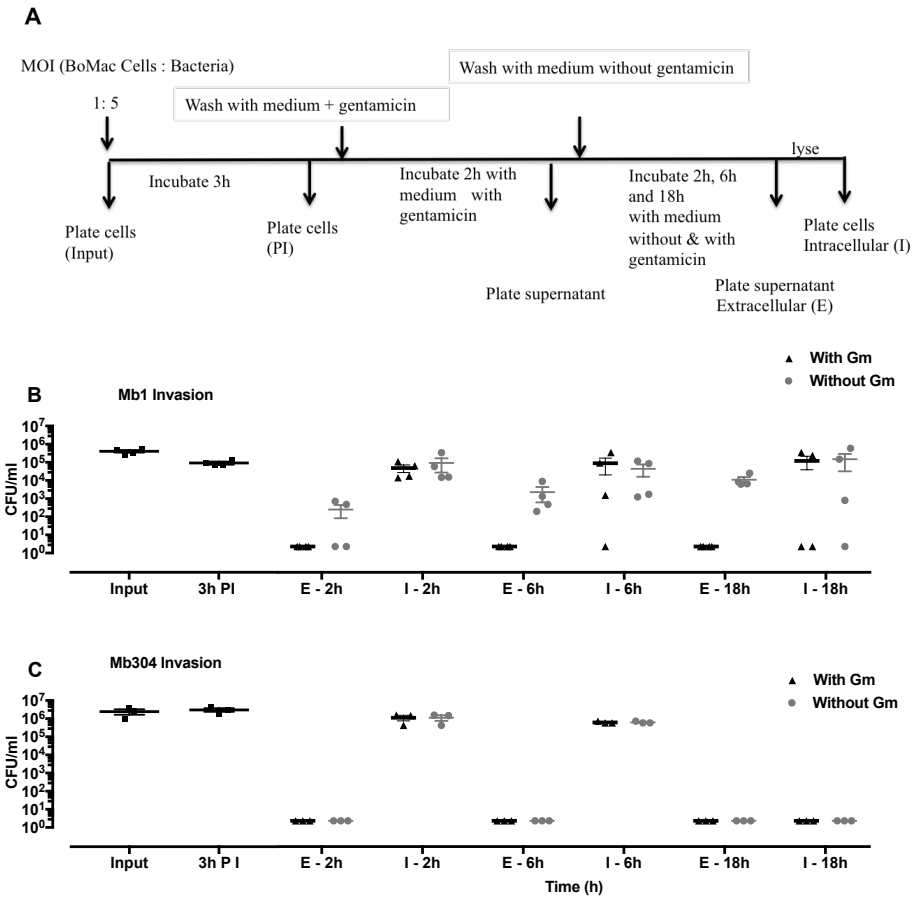


Figure 4. 1: Invasion and survival of *M. bovis* strains Mb1 (B) and Mb304 (C) in BoMac cells. Viable intracellular bacteria are shown at 2 h, 6 h, and 18 h post-Gm treatment (2h) using an MOI of 1:5. The input is the cfu/ml of bacteria used at the beginning of the experiment. The data shown are the mean duplicates values of 4 independent experiments in Mb1 and 3 independent experiments in Mb304 and standard errors of mean of the individual measurements are indicated as bars. The circle represents incubation without gentamicin while the triangle represents incubation in the presence of gentamicin. E: extracellular bacteria found in supernatant and I: intracellular bacteria recovered from cell lysate.

After 3h post-invasion (PI), both strains were recovered in relatively the same numbers in the cell lysate as the input cfu/ml used at the beginning of the experiment (Figs. 4.1a and b). There was no growth of bacteria in the culture supernatants following Gm treatment. No differences in intracellular bacteria recovery were observed in the presence or absence of Gm in both strains (Figs. 4.1a and b). There was recovery of viable intracellular bacteria from both strains 2 and 6h after the Gm treatment. In addition, there was no intracellular recovery of the bison isolate Mb304 at 18h of incubation (Fig. 4.1b) and no extracellular recovery at any time point suggesting that Mb1 is able to survive longer in BoMac cells and Mb304 cannot escape the macrophages. These findings indicated that *M. bovis* is capable of invading and persisting in BoMac cells as also shown in other cell types.

The *M. bovis* strain Mb1 decreases STS-induced apoptosis of BoMac cells

I used Alexa Fluor®488 annexin V/PI staining to assess the modulation of apoptosis in BoMac cells by both strains of *M. bovis*. Cells were treated with staurosporine, a known apoptosis inducer as a positive control and others left untreated as a negative control. I also selected *M. bovis* Mb304, a bison strain, which did not delay STS-induced apoptosis in bovine primary alveolar macrophages (Suleman et al., 2016a). I included live and dead bacterial cells to address the question if the delayed apoptosis effect was only observed with live cells. The results are shown in figure 4.2.

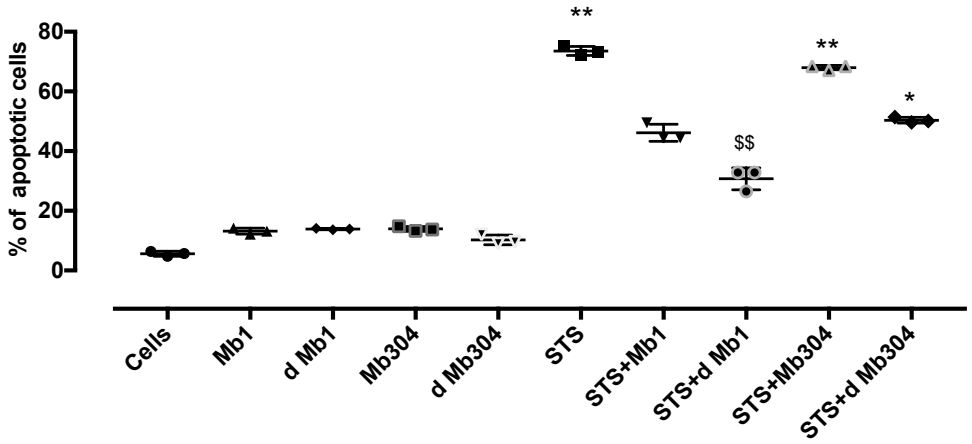


Figure 4. 2: Inhibition of BoMac cells apoptosis by *M. bovis* isolates from cattle and bison. Percentage of BoMac cells undergoing apoptosis after different treatments, live or dead (d) *M. bovis* strains were incubated with cells with or without STS. Controls included untreated cells and STS only treated cells. The bars show the mean percentages of apoptotic cells. Each data point is the mean of two technical replicates and the bars indicate the mean of three independent experiments. Significant differences between the treatments and untreated cells are indicated by * = $P<0.05$ or ** = $P<0.01$ and significant differences between the treatments and STS are indicated by $$ = $P<0.01$.

Compared to untreated cells, there was a significant increase in apoptosis after treatment with STS, STS+Mb1 and STS+Mb304 (6% vs. 73, 45, and 68% respectively). Compared to the untreated cells, there was no significant increase of apoptotic cells after treatment with dead or alive *M. bovis* Mb1 or Mb304 (Fig. 4.2). Compared to STS-treated cells, there was a 38% reduction of STS-induced apoptosis (from 73% to 45%) in BoMac cells treated with live Mb1+STS and a significant ($P<0.01$) 43% reduction after treatment with dead Mb1+STS (From 73% to 30%). Similar to Mb1, compared to untreated cells there was a 5% decrease (from 73% to 68%) of apoptotic cells in cells treated with live *M. bovis* Mb304. Incubation with killed Mb304 resulted in a 23% reduction of apoptotic cells (from 73% to 50%). Overall, these results are consistent with the delay in apoptosis by Mb1 and Mb304 previously observed in primary bovine alveolar macrophage (Suleman et al., 2016a).

M. bovis inhibits the activities of caspases 3, 6 and 9 in BoMac cells

Given the cardinal role of caspases in apoptosis, I investigated the modulation of BoMac STS-induced caspase activity following incubation with Mb1 and Mb304 and the results are shown in figure 4.1. There was no increase in caspases 3, 6, and 9 activities after infection with both *M. bovis* strains. Incubation with the protease inhibitor Z-VAD-FMK also resulted in no increases in

caspases activity (Fig. 4.3). Compared to untreated cells, there was a significant increase in caspases 3, 6, and 9 activities in cells treated with STS, Mb1+STS, and Mb304+STS. Compared to cells treated with STS, there was a significant reduction of STS-induced caspases 3, 6, and 9 in cells treated with Mb1+STS and Mb304+STS. The findings on *M. bovis* strain Mb1 are consistent with those found in bovine monocytes (Mulongo et al., 2014).

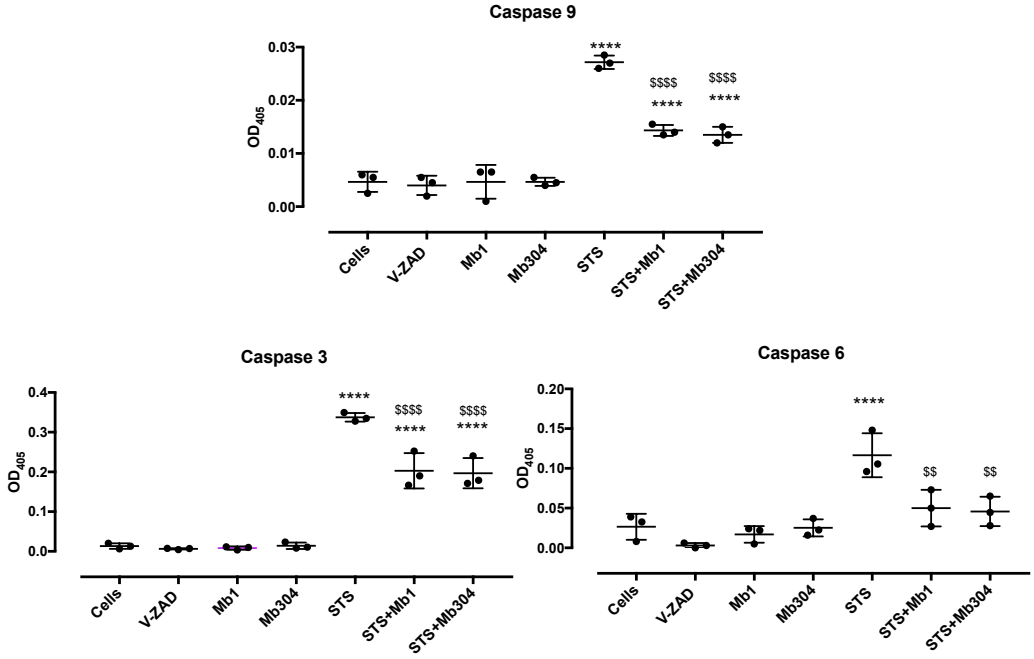


Figure 4. 3: Effect of *M. bovis* infection on BoMac cells caspase activity. Cells were treated with STS (6h), Mb1 and Mb304 (3 h), in the presence or absence of STS 6h post-infection, and V-ZAD-FMK an apoptosis inhibitor (40uM, 24h). Each column represents the average of duplicates of four independent experiments for each treatment. The bars represent the standard deviations of individual measurements. Significant differences between the treatments and untreated cells are indicated by *** = P<0.001 and **** = $P\leq$ 0.0001, significant differences between the treatments and STS are indicated by $$ = P<0.01, $$$$ = P<0.0001.

M. bovis modulates ROS production

ROS is crucial in immunological processes both as a component of the killing response of immune cells to microbial invasion and an initiator for the intrinsic apoptotic-signalling cascade (Fang, 2011; Redza-Dutordoir and Averill-Bates, 2016). I investigated if *M. bovis* modulated pyocyanin-induced ROS production and results are presented in figure 4.4. Compared to untreated cells, there was an increase in the production of ROS (P<0.001) after incubation with pyocyanin, confirming that ROS is induced in these cells (Fig. 4.4). No changes in ROS production were detected upon infection with either of the *M. bovis* strains alone compared to untreated cells. Compared to pyocyanin-treated cells, cells treated with pyocyanin and infected with Mb1, (Pyo+Mb1), showed a significant reduction (P<0.001) in ROS production (Fig. 4.4). In contrast, compared to pyocyanin-treated cells there was no significant reduction in ROS production in cells treated with pyocyanin and infected with Mb304 (Pyo+Mb304) (Fig. 4.4). These results indicate that the cattle *M. bovis* strain Mb1 inhibited pyocyanin-induced ROS production but the bison strain Mb304 failed to decrease ROS production in the presence of pyocyanin.

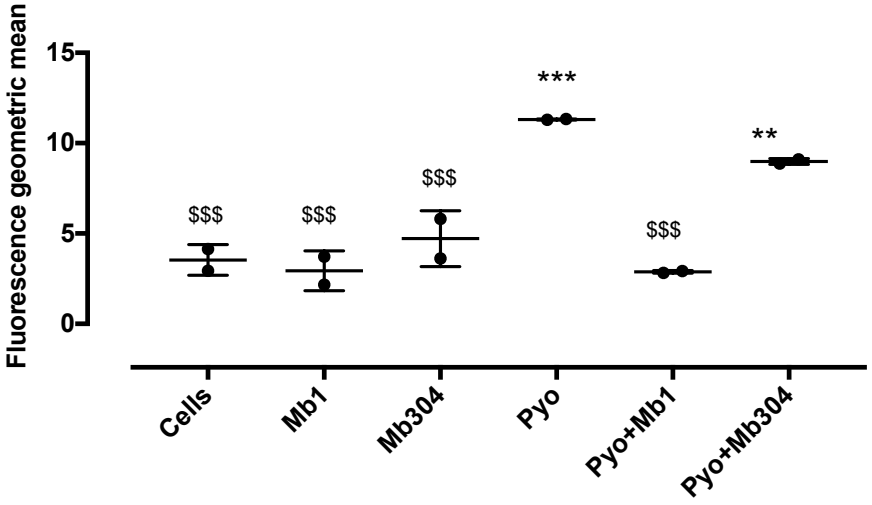


Figure 4. 4: Decrease in intracellular ROS after *M. bovis* infection of BoMac cells. BoMac cells were treated with both strains of *M. bovis* in the presence and absence of pyocyanin (Pyo). Pyocyanin alone and untreated cells were included as controls. FACS was used to analyse the data. The fluorescence geometric mean intensity is shown in the *Y* axis. Each column represents the average duplicates of two independent

experiments. Significant differences between the treatments and untreated cells are indicated by ** = $P<0.01$, *** = $P<0.001$ and significant differences between the treatments and pyocyanin are indicated by $$$ = $P<0.001$.

Mitochondrial cytochrome *C* release to the cytosol is decreased by *M. bovis* infection of BoMac cells

Cytochrome *C* translocation from the space between the inner and outer mitochondrial membranes to the cytosol allows for detection of changes in the intrinsic apoptotic pathway and is required for activation of caspase 9 (Kluck et al., 1997; Wang and Youle, 2009; Wang, 2001; Yang et al., 1997). I next asked whether *M. bovis* anti-apoptotic effects could prevent BoMac cytochrome *C* release to the cytosol during STS-induced apoptosis and the results are shown in figure 4.5. By comparing the mitochondrial fractions with that from cytosolic fractions, I found that there was decreased translocation of cytochrome *C* to the cytosol of un-infected and infected cells with both mycoplasma strains in the absence or presence of STS as shown in the Western blot compared to the increased translocation in STS-treated cells (Fig. 4.5a). These results were confirmed by semi quantitative band intensity analysis performed by the Odyssey® CLx Imaging System (Fig. 4.5b). This observation suggests that *M. bovis* decreases the release of cytochrome *C* to the cytosol, a common step required for the intrinsic apoptosis pathway.

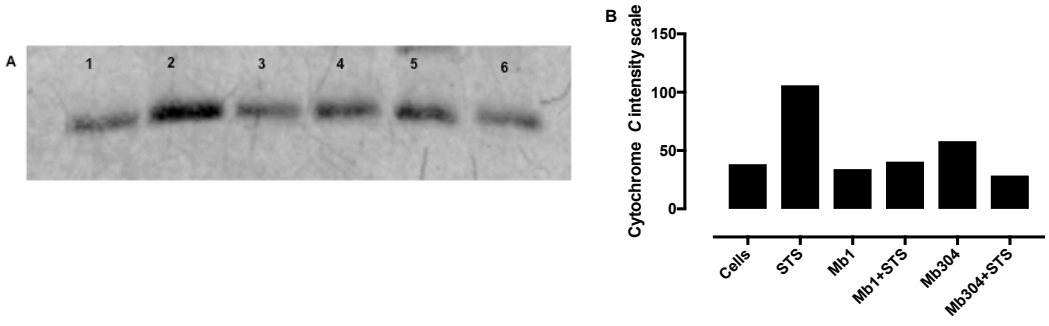


Figure 4. 5: Western blot analysis of *M. bovis* inhibition of mitochondrial cytochrome *C* release induced by staurosporine. BoMac cells were treated with both strains of *M. bovis* in the presence and absence of STS as described in Materials and Methods. (A) The cell samples were then fractionated and the cytosol fractions analyzed by western blots. 1: untreated cells; 2: STS-treated cells; 3: *M. bovis* Mb1 treated cells; 4: *M. bovis* Mb1+STS treated cells; 5: *M. bovis* Mb304 treated cells and 6: *M. bovis*

Mb304+STS treated cells. (B) Semi quantitative band intensity analysis performed by the Odyssey® CLx Imaging System.

M. bovis inhibits DNA fragmentation

The later stage of apoptosis is characterized by DNA fragmentation (Elmore, 2007); hence, I next aimed to investigate whether the anti-apoptotic effect of *M. bovis* on STS-induced apoptosis had any effect on decreasing DNA fragmentation and the results are shown in figure 4.6. DNA integrity was assayed by agarose gel electrophoresis. As observed in Fig. 4.6, DNA fragmentation was not seen in untreated cells (lane 1) and *M. bovis*-infected cells (lanes 2 and 4). There was a slight fragmentation observed in STS-treated cells incubated with Mb1 or Mb304 (lanes 3 and 5) but significant DNA fragmentation of the STS-treated cells as demonstrated by the tailing of the smear (lane 6) due to degradation by nucleases. These results indicate that both strains of *M. bovis* inhibited fragmentation and decreased DNA fragmentation in the presence of an apoptosis inducer, STS compared to STS treated cells.

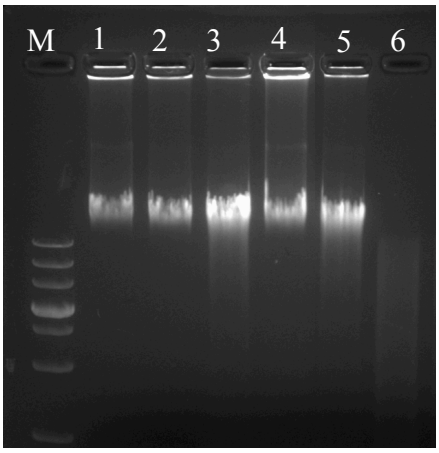


Figure 4. 6: DNA fragmentation analysis by agarose gel electrophoresis. DNA was prepared as described in Materials and Methods. DNA integrity was determined by horizontal agarose gel electrophoresis followed by staining with ethidium bromide. Lanes: M, molecular mass marker; 1: untreated cells; 2: *M. bovis* Mb1 treated cells; 3: *M. bovis* Mb1+STS treated cells; 4: *M. bovis* Mb304 treated cells; 5: Mb304+STS treated cells; 6: STS-treated cells.

NF-kβ -p65 nuclear translocation is activated in BoMac cells infected with *M. bovis.*

As previously reported for bovine monocytes cells (Mulongo et al., 2014) incubation with *M. bovis* Mb1 resulted in the translocation into the nucleus of the p65 subunit of NF-kβ. I investigated if the effect on NF-kβ was similar in BoMac cells incubated with Mb1 or Mb304 and the results are shown in figure 4.7. Western blots show that compared to the untreated cells; there was more nuclear translocation of the p65 subunit in LPS- and Mb1-treated BoMac cells (Fig. 4.7). Similarly, there was more translocation of p65 into the nucleus in cells treated with Mb304 (Fig. 4.7b). These results were confirmed by semi quantitative band intensity analysis performed by the Odyssey® CLx Imaging System (Fig. 4.7 A2-B2). There was no loading control in the Mb1 and Mb304 Western blots with LPS treatments (A and B respectively).

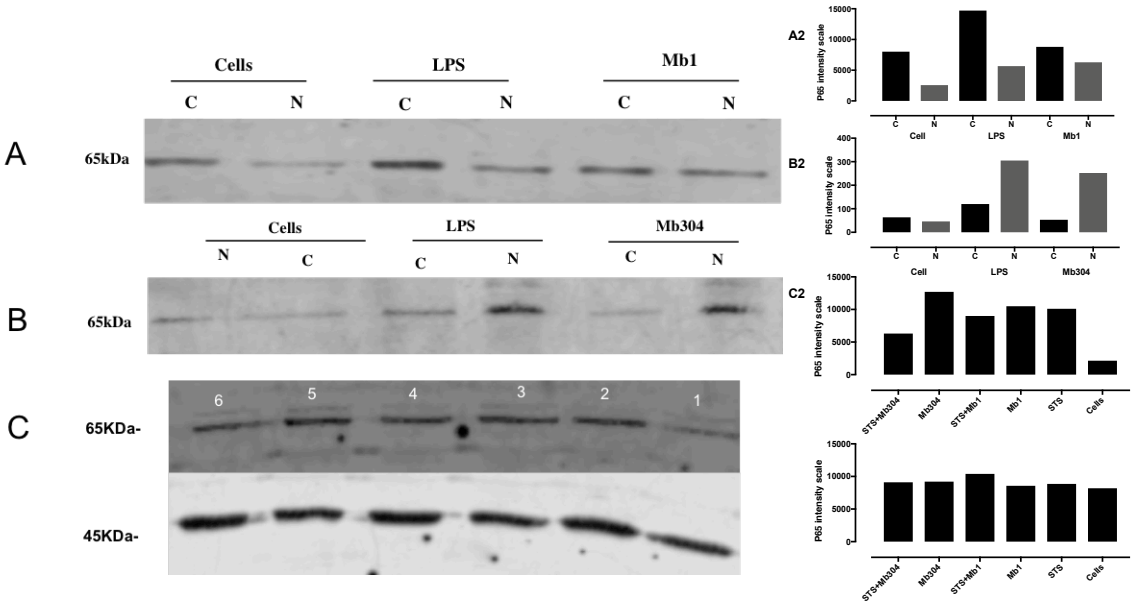


Figure 4. 7: Translocation of RelA (p65) NF-κB subunit from cytoplasm to the cell nucleus. In panel A and B cells were treated with 1 µg/ml LPS and Mb1 (A) and Mb304 (B) for 24 hours, followed by lysing of the cells, separation of the cytoplasmic (C) and nuclear (N) fractions, and probing with antibodies specific for p65. Untreated cells were used as a control. In panel C, only the nucleic fractions of the different treatments of the cells were analyzed with antibodies specific for p65 (65 kDa) and β-Actin (45 kDa) to ensure equal loading. Untreated cells (lane 1), cells treated with the apoptosis inducer 1 µg/ml STS for 6 h (lane 2), Mb1 24 h (lane 3), STS+Mb1 (lane 4), Mb304 24 h (lane 5) and STS+Mb304 (lane 6). (A2-C2) Band intensity was visualized by Odyssey® CLx Imaging System.

To determine the effect of STS on translocation of the NF-kβ p65 subunit, I incubated BoMac cells with STS, Mb1, STS+Mb1, Mb304 and STS+Mb304. Only the nucleus fractions were assayed on the Western blot. Compared to the untreated cells, the band intensities of the p65 nuclear fractions were higher in STS, Mb1, STS+Mb1, Mb304 and STS+Mb304 (Fig. 4.7C, lanes 2-6). These results were confirmed by semi quantitative band intensity analysis performed by the Odyssey® CLx Imaging System (Fig. 4.7 C2). The results show activation of NF-kβ in BoMac cells infected with both strains of *M. bovis* and simultaneously treated with STS.

Infection of BoMac cells with *M. bovis* induces the expression of anti-apoptotic genes, Bcl-X_L and Bcl-2

Translocation of the NF-kβ p65 protein into the nucleus of the cell results in regulation of expression of pro- and anti-apoptotic genes. I tested the effect of *M. bovis* in STS-treated and untreated BoMac cells gene expression of the *Bcl-X_L* and *Bcl-2* genes involved in anti-apoptosis and the *Bax* gene involved in the induction of apoptosis at 3h and 24h post-infection with both mycoplasma strains. The results are shown in figure 4.8. Relative to the GAPDH and β-actin levels, I observed a significant increase in the level of expression of the anti-apoptotic *Bcl-2* gene compared to the untreated cells in Mb1 (3h)- and Mb304 (3h)-infected cells and also an increase in expression at a later time point (24h) and in STS+ *M. bovis* although not significant compared

to the control. Treatment with STS alone showed no apparent differences in *Bcl-2* expression compared to the control. Similar results were observed with the *Bcl-X_L* gene. There was a significant increase of *Bcl-X_L* gene expression in Mb1 (3h), Mb304 (3h), and Mb304 (24h) but also an increase in expression after STS + *M. bovis* although not significant (Fig. 4.8). However, there were no significant differences in the expression of pro-apoptotic *Bax* gene in response to infection with either strain of the *M. bovis*, STS-treated cells and un-stimulated cells (Fig. 4.8).

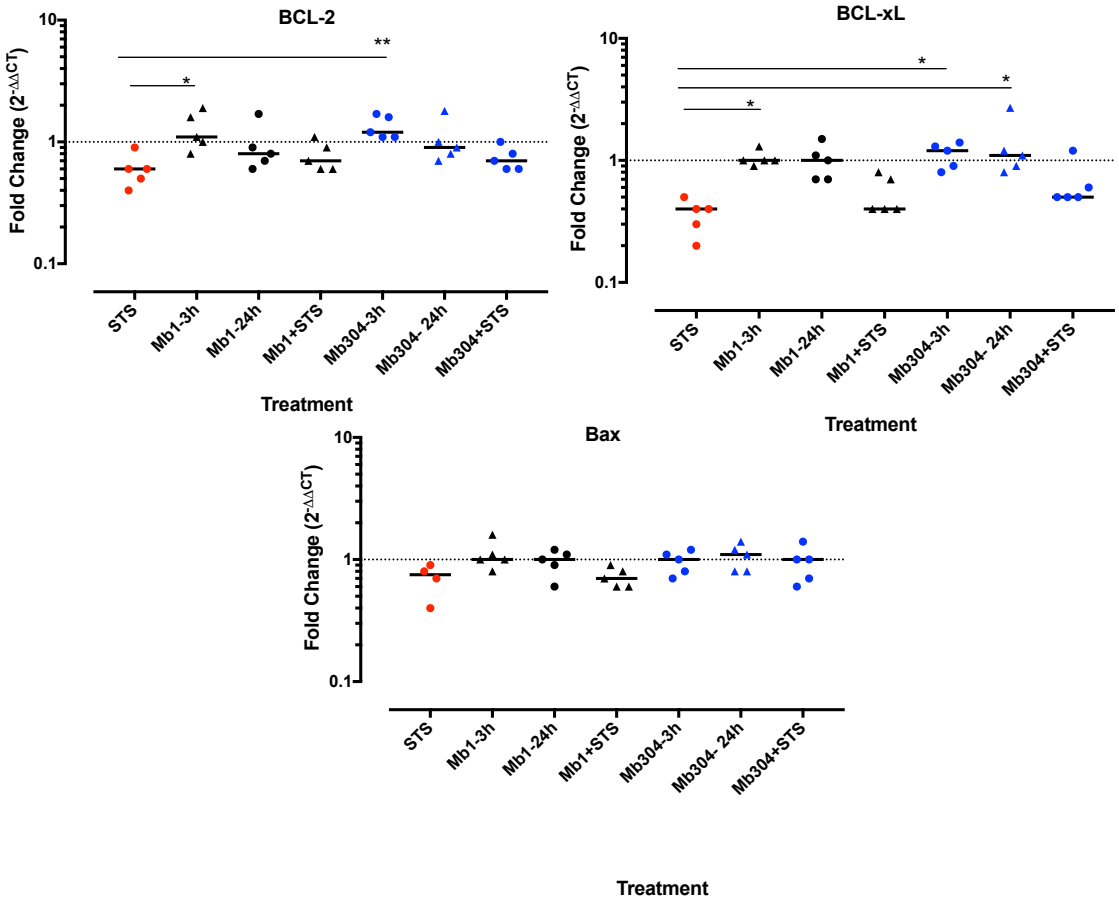


Figure 4. 8: *Bcl-X_L* and *Bcl-2* genes are upregulated in BoMac cells infected with *M. bovis* as determined by qPCR. RNA was isolated from BoMac cells infected with both strains in presence or absence of STS. Each data point shown represent the average duplicates of five independent measurements. Significant differences between the treatments and untreated cells are indicated by * $P<0.05$ and ** $P<0.01$.

4.4. Discussion

Mycoplasma invasion of the lungs attracts monocytes, macrophages and neutrophils creating an inflammatory milieu that attempts to localize and eliminate the infection (Hermeyer et al., 2012a; Hermeyer et al., 2011; Howard and Taylor, 1983; Howard et al., 1976). Nonetheless, there is evidence of mycoplasma persistence in both normal and diseased lungs, suggesting the existence of mechanisms by which the bacterium can evade this inflammatory assault (Hermeyer et al., 2012a; Hermeyer et al., 2011; Khodakaram-Tafti and Lopez, 2004; Rodriguez et al., 1996).

Induction (Lancellotti et al., 2009) or inhibition (Faherty and Maurelli, 2008) of apoptosis has been reported in different pathogens and cell types and plays a vital role in disease pathogenesis (Lancellotti et al., 2009; Faherty and Maurelli, 2008). Our laboratory has previously demonstrated delay of apoptosis in all PBMC subsets (van der Merwe et al., 2010), monocytes (Mulongo et al., 2014) by *M. bovis* including primary bovine macrophages (Suleman et al., 2016a) and the goal of the current study was to use a bovine macrophage cell line to characterize the mechanisms underlying this inhibition of apoptosis in order to identify potential pathways that could be targeted to achieve macrophage activity against mycoplasma. The bovine macrophage cell line (BoMac) is an *in vitro* differentiated bovine peritoneal macrophage cell line (Stabel and Stabel, 1995). It has been used to study infections with bovine herpesvirus-4 (BHV-4), *Map* (Langelaar et al., 2005; Souza et al., 2007; Woo et al., 2006) and *M. bovis* co-infection with bovine viral diarrhea virus (Burgi et al., 2018). In these different studies the cell line has shown the ability to develop *bona fide* functions of alveolar macrophages including the ability to phagocytize bacteria with and without opsonization, generate reactive oxygen species (ROS), produce different cytokines (IL-6, TGFβ-1, TNF-α, IFN-γ, IL1-α) and upregulate genes related to apoptosis (Bcl2-1) following activation and infection (Stabel and Stabel, 1995; Tooker et al., 2002). In a different study using this cell line, Abendaño showed an increased level of expression of the apoptotic inhibitor Bcl2-1 and low level of apoptosis and increased TGF-β after infection with *Map* (Abendano et al., 2013).

I first tested if our strains were capable of invading and surviving in the BoMac cell line at different multiplicity of infection (MOI) and with different infection time points using the gentamicin protection assay. These findings confirmed that there was recovery of viable intracellular Mb1 after 18h post-invasion and Mb304 after 6h post-invasion in presence and

absence of gentamicin (Fig. 4.2) at the different MOIs. Consistent with previous observations that gentamicin displays minimal eukaryotic cell membrane penetration (Elsinghorst, 1994), this antibiotic did not affect the recovery of intracellular bacteria and the viability of the cells throughout the experiment. The recovery of viable intracellular mycoplasma demonstrates the ability of both *M. bovis* strains, Mb1 and Mb304 to invade and persist in BoMac cells differently. The Mb304 bison strain did not escape the macrophage to the medium, as there was no isolation of extracellular (E) viable bacteria in medium without GM in any of the time points. In addition, there was less persistence of intracellular (I) Mb304 after 6h compared to Mb1 the cattle strain. These discrepancies could be due to the fact that the cell line used is of cattle origin and Mb1 is better adapted to it that the bison isolate, Mb304. Previous studies by Pilo's group also reported invasion and survival in BoMac cells of different cattle strains of *M. bovis,* strains JF4278 and L22/93 (Burgi et al., 2018).

I further explored if both our strains (Mb1 and Mb304) were capable of inhibiting apoptosis in BoMac cells as previously demonstrated by our group in primary bovine alveolar macrophages (Suleman et al., 2016a). Indeed, as shown previously, the cattle strain *M. bovis* Mb1 reduced STS-induced apoptosis in contrast to the bison strain, *M. bovis* Mb304 (Fig. 4.3) that did not. On the contrary, the study from the co-infection experiment model of *M. bovis* with bovine viral diarrhea virus (BVD) using BoMac cells reported that the *M. bovis* strain JF4278 caused slight apoptosis and delay in STS-induced apoptosis was not observed with the type of test they used (Burgi et al., 2018). These discrepancies may also reflect the diversity that exists within *M. bovis* strains as previously described in adherence to various host cell lines that influence virulence (Thomas et al., 2003a).

Successful bacterial pathogens have evolved different strategies to modulate apoptosis of various immune cells for survival and replication (Faherty and Maurelli, 2008). I further investigated which of the apoptotic pathways in macrophages was affected by infection with *M. bovis*. Different pathogenic bacteria modulate apoptosis by either protecting the mitochondrial integrity (Massari et al., 2000; Massari et al., 2003), preventing the release of cytochrome *C* and inhibition of caspase activation (Fan et al., 1998); or activating cell survival pathways by up-regulating inhibitors of apoptosis (Abendano et al., 2013; Binnicker et al., 2003; Goebel et al., 2001; Sukumaran et al., 2004).

Caspases are cysteine proteases that are initially synthesized as inactive pro-caspases that are cleaved to an active form upon activation by apoptotic stimuli. Caspases are divided into initiators caspases (2, 8, 9, and 10) and executioner caspases (3, 6, and 7) (McIlwain et al., 2013). I observed a decrease in protease activity of caspases 3, 6, and 9 STS-treated cells after infection with both strains of *M. bovis* compared to STS-treated cells and reduced activity in a similar trend as the caspase inhibitor (Fig. 4.3). In contrast, previous reports show that *M. bovis* strain Mb1 inhibits STS-induced apoptosis in monocytes by inhibiting caspase 9 but not 3. The results for caspase 6 in monocytes were inconclusive as there were no differences between untreated vs. Mb1-treated monocytes and a slight difference in Mb1 vs. STS-treated monocytes (Mulongo et al., 2014). The different observations may be attributed to the specific cell types (monocytes vs. macrophages) used for the assay. The mechanism of apoptosis of *M. bovis* has been shown to be dependent on caspase 9, an initiator caspase of the mitochondrial apoptosis pathway followed by activation of caspases 3 and 6, effector caspases that lead to DNA fragmentation and eventually cell death. Upon receiving an apoptosis signal, caspases 3 and 6 exert their effects downstream of initiator caspase 9 (Porter and Janicke, 1999), and would be expected to be inactive upon inhibition of apoptosis.

NF-kβ is a transcription factor responsible for gene expression of target genes, the majority of which participate in the host immune response and cell death. Defects in NF-κβ signalling result in increased susceptibility to apoptosis leading to increased cell death. NF-κβ regulates apoptosis by activating the expression of apoptotic or anti-apoptotic genes (Kucharczak et al., 2003). Usually, p65 is stored in an inactive form in the cytoplasm as part of the p65-p50 (NF-κβ) complex bound to the inhibitory protein IκBα. On activation, IκBα is phosphorylated by the IκB kinase leading to ubiquitination and release of the inhibitory protein from the NF-kβ complex. This, in turn, exposes nuclear import factors that target p65 to the nucleus where it binds to regulatory elements and modulates gene transcription (Rahman and McFadden, 2011). In bovine monocytes, NF-kβ activation following *in vitro* infection with *M. bovis* was measured by detection of accumulated p65 NF-kβ subunit in the nuclear fractions of infected cells compared to un-infected cells (Mulongo et al., 2014). Using the same assay, I observed activation of the NF-kβ signalling in BoMac cells following infection with both *M. bovis* Mb1 and Mb304 (Fig. 4.7).

Apoptosis induced by ROS production, the intrinsic pathway, leads to various events that occur in the mitochondria that include the loss of membrane potential ($\Delta\Psi_m$), cytochrome *C* release, and participation of the Bcl-2 homologs (Redza-Dutordoir and Averill-Bates, 2016; Wang and Youle, 2009; Wang, 2001). Apoptosis is regulated by two groups of modulators: the anti-apoptotic Bcl-2-family proteins (such as Bcl-X_L and Bcl-2), and pro-apoptotic family of proteins (such as Bax and Bak) (Elmore, 2007; Riedl and Shi, 2004; Shi, 2002). The pro-apoptotic proteins are mostly found in the cytosol and the anti-apoptotic proteins are localised in the outer mitochondrial membrane as heterodimers with apoptotic proteins (Bax and Bad) thus inhibiting their apoptotic functions and regulating mitochondrial $\Delta\Psi_m$, cytochrome *C* release, and caspase activation (Cory and Adams, 2002). In the present study, I observed suppression of ROS production by *M. bovis* strain Mb1 (Fig. 4.4) as also shown in leukocytes infected with *N. gonorrhoeae* (Chen and Seifert, 2011). These findings agree with previous findings that observed no increase in neutrophil intracellular ROS levels upon stimulation with *M. bovis* (Gondaira et al., 2017; Mitiku et al., 2018; Redza-Dutordoir and Averill-Bates, 2016; Thomas et al., 1991). Host cells generate ROS as part of the oxidative burst during an infection to control microbial infection and its role in DNA damage during apoptosis is described in the literature (Simon et al., 2000; Wang and Youle, 2009). In addition to inhibition of ROS, I also report reduced translocation of cytochrome *C* from the mitochondrial intermembrane space to the cytosol of infected BoMac cells (Fig. 4.5). Again, our results are consistent with previous reports in *N. gonorrhoeae* (Chen and Seifert, 2011), suggesting that *M. bovis* inhibits apoptosis in BoMac cells by preventing mitochondrial depolarization. The anti-apoptotic Bcl-2 family of proteins, maintain the $\Delta\Psi_m$ of the mitochondria, and prevent cytochrome *C* release to the cytoplasm and hence, inhibit activation of initiator caspase 9 that exerts its effects upstream of executioner caspases 3 and 6 (Sukumaran et al., 2004). I subsequently report an up-regulation of the anti-apoptotic genes, Bcl-X_L and Bcl-2 by both strains in BoMac cells (Fig. 4.8). These results are consistence with a study that reported up-regulation of the *Bcl-2* gene in BoMac cells infected with *Map* (Abendano et al., 2013). In *E. coli* infection of RAW 264.7 cell line induces the expression of Bcl-X_L (Sukumaran et al., 2004), and with *Toxoplasma gondii* infection of U937 cells induces expression of Mcl-1, another anti-apoptotic protein of the Bcl-2 family (Goebel et al., 2001) and in *Neisseria spp* they reported up-regulation of c-IAP-2, and Mcl-1 in primary human urethral

epithelial cells (Binnicker et al., 2003). Hence, I cannot exclude the involvement of additional anti-apoptotic member of the Bcl-2 protein family and IAPs.

4.5. Conclusion

Taken together, our findings suggest novel mechanisms that *M. bovis strain* Mb1 reduces STS-induced apoptosis via the intrinsic pathway in a caspase dependent manner by inhibition of caspases 3, 6, and 9; decreased cytochrome *C* release; activation of NF-β with anti-apoptotic consequences of increased up-regulation of the anti-apoptotic survival genes *Bcl-*X_L and *Bcl-2* and absence of DNA fragmentation. With the bison strain, *M. bovis* Mb304 I observed similar results except for the STS-induced apoptosis assay where Mb304 induced significantly less reduction in apoptosis reported in this thesis (Fig. 4.2) and in primary alveolar macrophages (Suleman et al., 2016a). These discrepancies between the two isolates (cattle isolate Mb1 and bison isolate Mb304) can be attributed to the difference in strain specific variations and evolutionary adaptation to the different host.

CHAPTER 5. General discussion and conclusions

5.1. Summary

The general purpose of this thesis was to study host-pathogen interactions specifically, the modulation of macrophages effector functions and apoptosis, following infection by *M. bovis*.

Since mycoplasmosis is highly dependent on the *M. bovis* ability to subvert the macrophage innate immune response I sought to characterize the interaction between *M. bovis* and bovine macrophages. As outlined in this thesis, Mb1 has evolved multiple strategies to survive and disseminate within the bovine host.

There are several studies (including this, Chapter 4) that outline invasion and survival of *M. bovis* in bovine macrophages as an immune evasion tactic *in vitro* (Burgi et al., 2018; Maina et al., 2018; Suleman et al., 2016a) and *in vivo* (Adegboye et al., 1995; Kleinschmidt et al., 2013). Evidence of *M. bovis* as an intracellular bacterium allows it to hide from humoral mediated immune responses and some antimicrobial therapy. The ability of the immune cells to move within the host also allows an interesting way for pathogens to disseminate systemically inside their host to various tissues such as, synovial membrane (Haines et al., 2001b), liver (Adegboye et al., 1995), and kidneys (Hermeyer et al., 2012b).

Other strategies for immune evasion by *M. bovis* include manipulation of macrophage effector functions such as cytokine production by eliciting an anti-inflammatory IL-10-skewed cytokine profile at the expense of the pro-inflammatory TNF-α cytokine profile. IL-10 is known to affect the APCs by inhibiting their ability to release pro-inflammatory cytokines and can also directly affect T-cells by suppressing proliferation and production of cytokines (Couper et al., 2008b). These results are consistent with the earlier observation in monocytes where there was reduced production of TNF-α and IFN-γ and an increase in IL-10 cytokine (Mulongo et al., 2014). Production of pro-inflammatory cytokine causes infiltration of leucocytes and other immune cells in the lungs, which eventually contributes to the elimination of the pathogen, but if left unchecked can also cause clinical pathology.

In this thesis, I also explored the modulation of nitric oxide production by infected primary BAMs as an immune evasion strategy. NO is an efficient way of controlling intracellular

pathogens by phagocytic cells by direct NO action or as a key pro-inflammatory signalling molecule. I showed that *M. bovis* strain Mb1was able to reduce production of NO in primary BAMs. In other bacteria such as intracellular *Salmonella,* Type 3 secretion system is used to mediate the NO modulation (Chakravortty and Hensel, 2003). In this thesis, I did not investigate the mechanism by which *M. bovis* attenuates iNOS expression and NO production.

While apoptosis is considered important in to host defence mechanism bacteria have evolved strategies to manipulate host cell death mechanisms for their survival, multiplication as well as dissemination (Renehan et al., 2001). The ability of bacteria to induce or inhibit apoptosis can profoundly alter the immune response resulting in successful host invasion. Hence, I also explored in this thesis the effect of *M. bovis* strain Mb1 on macrophages apoptosis and the mechanism of inhibition for survival in the host. Indeed, in this thesis, I was able to show the different mechanisms of apoptosis that are modulated during inhibition of apoptosis (Chapter 4). As a result of these studies, I have proposed a novel mechanism by which *M. bovis* prolongs the life of infected bovine alveolar macrophages by inhibiting the intrinsic apoptosis pathway. Mb1 is able to inhibit activation of caspases 3, 6, and 9, protecting the mitochondrial integrity by preventing the release of cytochrome *C*, inhibiting DNA fragmentation, activation of NF-β and activating cell survival pathways by up-regulating anti-apoptotic survival genes *Bcl-*X_L and *Bcl-2*. By prolonging the life of the immune cell, the bacterium ensures survival and systemic dissemination in the host.

It is also now apparent that different strains of *M. bovis* differ in their invasion, pathogenicity and immune modulation in various cell types (Burgi et al., 2018; Thomas et al., 2003a). Herein, I observed some different effects while using a bison isolate *M. bovis* strain Mb304 in the apoptosis experiment. Mb304 did not decrease STS-induced apoptosis in BoMac cells (Chapter 4) and primary cattle alveolar macrophages (Suleman et al., 2016a) and did not reduce ROS production by BoMac cells.

Understanding the factors that contribute to virulence, dissemination and immune evasion of a pathogen is critical knowledge for contemplating new vaccines and therapeutics.

5.2. Challenges, limitations and prospects

The use of primary cells in the earlier studies (chapter 2) was a challenge as the data derived from these studies had large dispersion. The cost and logistics associated with large animal studies was a limitation and the lack of a small animal model for *M. bovis* or cell line at that point was a challenge.

Another gap in the mycoplasma field is the shortage of genetic tools like those found for other bacteria, but there have been some current improvements. In respect to *M. bovis* the development of fluorescence expression tools to study host-mycoplasma interactions resulted in the construction of two strains expressing the mNeonGreen and mCherry proteins (Bonnefois et al., 2016). In addition, genetic manipulation using the transposon Tn4*001* (Chopra-Dewasthaly et al., 2005) and plasmids for gene expression (Li et al., 2015; Sharma et al., 2015) have been developed.

In the future, more studies are needed to assay for modulation of other BAMs effector functions such as receptor-mediated phagocytosis with the aid of new technologies such as the use of fluorescence mycoplasma cell-labelling already developed for investigating cell invasion. To produce the mNeonGreen and mCherry high-expression mycoplasma strains they also used transposon mutagenesis with a Tn4*001*-derivative mini-transposon (Bonnefois et al., 2016). To understand cell invasion, real-time cell infections can now also be investigated, and multiple fluorescent markers can be used to tag different mycoplasmas, strains or species to analyse mixed infections. This technology is not limited to *in vitro* use but it is also ideally suited for use *in vivo* by direct imaging of small animal models or hosts as reported in other bacteria (Zelmer et al., 2012).

More needs to be understood about *M. bovis* pathogenesis. I used in this thesis the cattle isolate *M. bovis* strain Mb1. Other strains with different virulence properties or field isolates need to be used for comparison studies. The disparate finding highlighted in the apoptosis study in chapter 4 between the cattle and bison isolate (Mb1 and Mb304 respectively) shows the need for further comparisons of field isolates. Also In chapter 4, there was a limitation in the lack of a loading control of the western blots assay performed for both NF-kβ P56 molecule and cytochrome *C* translocation to ensure accurate loading of the proteins in the gel and the assay should have been

repeated severally to allow semi quantification of the band intensity analysis using the Odyssey® CLx Imaging System.

In chapter 3, I investigated the ability of infected primary BAMs to present antigens to T-cells. In that study, I was not able to prove that the calves were infected and did not use any other known antigens. Further investigations are required to provide detailed information on how possible differences on infectious dose, choice of recall antigen, host susceptibility, route of infection, infection timeframe, breeds and age of the calves could affect reproducibility of the disease model. Also, an increase in the experimental sample size should be considered.

The genome sequence of *M. bovis* Mb1 is unknown. The availability of genome sequences of different *M. bovis* isolates will, in turn, improve diagnostics and epidemiological traceability of the pathogen through comparative genomic studies. Very few studies in *M. bovis* correlate specific genes with pathogenicity (Rasheed et al., 2017), and so far to date, no gene has been identified that influences *in vivo* virulence of any *M. bovis* isolate.

Furthermore, dissecting the molecular mechanisms behind inhibition of apoptosis and modulation of T-cell proliferation in the host may also allow us to predict the factors that interfere with the host-pathogen interactions, leading to increased persistence, infection and disease. The biological significance of the data generated in my thesis on apoptosis remains to be tested in an animal model. In the future, the unique adaptations by *M. bovis* that have been identified in this thesis can be tested in a relevant model. Further studies are needed to investigate if inhibition of apoptosis is associated with less or more virulent strains of *M. bovis* as shown in *Mycobacterium spp.* The virulent strains were shown to inhibit apoptosis while attenuated strains enhanced apoptosis in macrophages. These findings led to mutation of different virulennce genes and an increase of macrophage apoptosis was observed (Velmurugan et al., 2007). Hence, if the genetic formation of a bacterium influenced the mechanism of apoptosis then, this would make a huge contribution in creating attenuated vaccine strains. The discovery of genes responsible for apoptosis modulation by *M. bovis* would provide an opportunity to understand the host-pathogen interaction further and inform the pathogenesis of the disease.

Manipulation of the macrophage activation response to *M. bovis* involving pathogenesis aspects has been proposed as a promising new avenue for effective development of vaccines strategies

and treatment of infection. We demonstrated (I as a co-author) that when we blocked the PD-1/PD-L1 immune checkpoint with a blocking anti PD-1 antibody we restored proliferation of Mb1-infected PBMC (Suleman et al., 2018), while other studies demonstrated restoration of IFN-γ production in PBMCs in bovine mycoplasmosis (Goto et al., 2017). Thus, these findings can be used as a supplement to regular vaccine as antibody-mediated therapy to prevent disease progression.

Just as a kinome array was used previously to study bovine monocytes and *M. bovis* interactions (Mulongo et al., 2014) other robust techniques such as RNA-seq (Wang et al., 2009) for host-pathogen interactions should be explored in the future. This would provide information about significantly up- or down-regulated genes involved in the host-pathogen interactions as carried out in infection of sheep mammary glands with *M. agalactiae* (Chopra-Dewasthaly et al., 2017).

Further studies are needed to determine the best approach to prevent or arrest *M. bovis* ability to manipulate macrophage effector functions and enable better methods to combat mycoplasmosis.

Appendix

Appendix A: Tables

Table A. 1: List and concentration of coupled beads, standards and antibodies used in the Bioplex ELISA.

Cytokine standards		
Cytokine	**Catalogue number**	**Concentration (ng/ml)**
IL-10	Kingfisher	10
IL-1	Cyanamid	0.5
IL-12	Kingfisher RP0077B-005	20
IL-17	Kingfisher RP0056B-005	2
IL-6	Kingfisher RP0014B-005	10
IL-13	Kingfisher RP0002B-005	10
IFN -α	In house	0.5
IFN-γ	In house	1
IL-2	Cyanamid PRCB52692	20
IL-4	Biorad PBP006	0.1
IL-8	Kingfisher RP0023B-005	1
Coupled beads		
Cytokine	**Catalogue number**	**Bead region**
IL-10	Serotec MCA2110	26
IL-1	Serotec AHP851Z	62
IL-12	Serotec MCA1782EL	37

IL-17	Kingfisher PB0274B-100	43
IL-6	Kingfisher KP0652B-100	29
IL-13	Kingfisher PB0120B-100	52
IFN -α	In house 1C6+adh IgG	45
IFN-γ	In house 2-2-1A IgG	65
IL-2	VMRD clone 14.1	36
IL-4	Biorad MCA2371	55
IL-8	Kingfisher PB1164B-100	28
Detection antibodies (all biotinylated)		
Cytokine	**Catalogue number**	**Concentration (µg/ml)**
IL-10	Serotec MCA 2111B	2.5
IL-1	Serotec AHP851B	1
IL-12	Serotec MCA2173B	2.5
IL-17	Kingfisher PBB0277B-050	0.2
IL-6	Kingfisher KPB0653B-050	1
IL-13	Kingfisher PBB0153B-050	0.1
IFN-γ	In house 92-133	1/250
IFN -α	In house 92-131 or 92-132	1/2000
IL-2	R&D baf2465	0.1
IL-4	Biorad MCA2372B	2
IL-8	Kingfisher PBB1165B-050	0.1

Table A. 2: Antibodies dilutions used in the flow cytometry assay of BAMs surface marker.

Antibodies	Dilution	Company
Mouse anti human macrophage APC	1/200	Serotec/Biorad
Mouse anti bovine CD40-RPE	1/200	Serotec/Biorad
Mouse anti bovine CD80-RPE	1/100	Serotec/Biorad
Mouse anti pig CD163-PE	1/100	Serotec/Biorad
Mouse anti bovine CD86-FITC	1/100	Serotec/Biorad
Mouse anti bovine CD205-FITC,	1/100	Serotec/Biorad
Mouse anti bovine CD11b-FITC	1/200	Serotec/Biorad
MHC class II DR isotype IgG1	1/100	VMRD/Kingfisher
Secondary antibody IgG1-PE	1/100	Serotec/Biorad

Appendix B: Co-authored papers and my contributions

Title: *Mycoplasma bovis*-induced inhibition of bovine peripheral blood mononuclear cell proliferation is ameliorated after blocking the immune-inhibitory programmed death 1 receptor

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Citation

Suleman, M., Cyprian, F.S., Jimbo, S., **Maina, T**., Prysliak, T., Windeyer, C., Perez-Casal, J., 2018. *Mycoplasma bovis*-Induced Inhibition of Bovine Peripheral Blood Mononuclear Cell Proliferation Is Ameliorated after Blocking the Immune-Inhibitory Programmed Death 1 Receptor. Infection and Immunity 86.

My contributions

I contributed in the sample and data collection and analysis on BAMs, and participated in manuscript review.

Modified abstract

We have previously shown that down-regulation of lymphocyte proliferation is another important immunosuppression strategy of *M. bovis* infection (Mulongo et al., 2013; Prysliak et al., 2013; van der Merwe et al., 2010; Vanden Bush and Rosenbusch, 2003). We suggested that the expression of PD-1 and PD-L1 played a role in the impairment of antigen-specific T-cell responses. The PD-1 receptor is expressed on activated T-cells and recognizes its ligand, PD-L1 expressed on activated dendritic and macrophages cells and engagement of both leads to poor immune responses. In the study, we used EBTr cells, EBL cells, and BAMs cells to investigate modulation of PD-L1 after *M. bovis* infection and the PD-1-related immune exhaustion of T-cells

during *M. bovis* infection. We showed an increase in the expression of the PD-1 receptor on total PBMCs specifically $CD4^+$ and $CD8^+$ positive cells and increased PD-L1 expression on purified lung lavage macrophages after Mb1 infection. We also blocked PD-1 receptors on PBMCs using anti-PD-1 antibody at the beginning of infection and demonstrated restoration of *M. bovis* infected PBMCs proliferation (Suleman et al., 2018).

Title: Effect of *Mycoplasma bovis* on bovine neutrophils

Citation

Jimbo, S., Suleman, M., **Maina, T**., Prysliak, T., Mulongo, M., Perez-Casal, J., 2017. Effect of *Mycoplasma bovis* on bovine neutrophils. Veterinary Immunology and Immunopathology 188, 27-33.

Copyright

My contributions

I generated the nitric oxide assay data and participated in manuscript review.

Modified abstract

We know that activation of macrophages leads to the recruitment of neutrophils to the site of infection (Gagea et al., 2006b). The aim of the study was to investigate the modulation of specific neutrophil activities such as oxidative bursts after infection with Mb1. We hypothesised that Mb1 modulates neutrophil functions and apoptosis to support its persistence and systemic dissemination. We showed that Mb1 had no effect on pro-inflammatory cytokines, IL-12 and TNF-α production; there was an increase in elastase production, induction of apoptosis and an inhibition of NO production. These results show different mechanisms by which *M. bovis* infections persist systemically and in the bovine lung (Jimbo et al., 2017).

Title: Status of the development of a vaccine against *Mycoplasma bovis*

Citation

Perez-Casal, J., Prysliak, T., **Maina, T**., Suleman, M., Jimbo, S., 2017. Status of the development of a vaccine against *Mycoplasma bovis*. Vaccine 35, 2902-2907.

Copyright

My contributions

I participated in the manuscript review.

Modified abstract

We have reviewed the status of different vaccines used in *M. bovis* control to-date that include bacterin-based vaccines, protein-based and live-attenuated vaccines. Additionally, we also highlighted the gaps limiting effective vaccines strategies for *M. bovis* (Perez-Casal et al., 2017).

Title: Th-17 cell mediated immune responses to *Mycoplasma bovis* proteins formulated with Montanide ISA61 VG and curdlan are not sufficient for protection against an experimental challenge with *Mycoplasma bovis*

Citation

Prysliak, T., **Maina, T**., Perez-Casal, J., 2018. Th-17 cell mediated immune responses to *Mycoplasma bovis* proteins formulated with Montanide ISA61 VG and curdlan are not sufficient for protection against an experimental challenge with *Mycoplasma bovis*. Veterinary Immunology and Immunopathology 197, 7-14.

Copyright

My contributions

I assisted in the collection and processing of samples during the animal trial. I also participated in the manuscript review

Modified abstract

In the literature Th-17 cells have been implicated in protection against respiratory pathogens by production of IL-17 and recruiting neutrophils. The aim of the study was to investigate if there were any *M. bovis* antigens that could elicit production of IL-17. The *M. bovis* antigens included recombinant proteins previously published, membrane fractions and whole cell extracts formulated in curdlan adjuvant, an inducer of Th-17 responses and Montanide™ ISA 61VG adjuvant. We showed significant humoral and cell mediated responses to the antigens; however, there was no protection in vaccinated animals against an Mb1 infection (Prysliak et al., 2018).

Title: Analysis of immune responses to recombinant proteins from strains of *Mycoplasma mycoides* subsp. *mycoides*, the causative agent of contagious bovine pleuropneumonia.

Citation

Perez-Casal, J., Prysliak, T., **Maina, T**., Wang, Y., Townsend, H., Berverov, E., Nkando, I., Wesonga, H., Liljander, A., Jores, J., Naessens, J., Gerdts, V., Potter, A., 2015. Analysis of immune responses to recombinant proteins from strains of *Mycoplasma mycoides* subsp. *mycoides*, the causative agent of contagious bovine pleuropneumonia. Veterinary Immunology and Immunopathology 168, 103-110.

Copyright

My contributions

I assisted in protein expression and purification, vaccine formulation and sterility testing, processing of samples as well as analysis of the data and participated in the manuscript review.

Modified abstract

We identified 66 possible vaccine candidates using reverse vaccinology using available *Mycoplasma mycoides* subsp. *mycoides (Mmm)* genome data. We expressed and purified the potential surface exposed protein vaccine candidates. We ranked the proteins based on their ability to be recognized by serum from CBPP-positive cattle and vaccinated cattle. We showed that the proteins tested were able to elicit good IgG1and IgG2 humoral responses. However, none of the recombinant proteins were able to elicit a recall response in PBMCs (Perez-Casal et al., 2015).

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